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Applicants: Jane Ellen Visvader, et al.

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Sir:

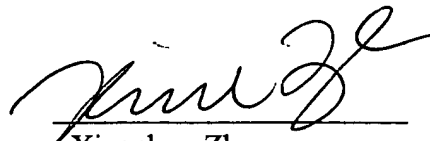
Applicants submit herewith a certified copy of the provisional patent application No.

PR7618 filed on September 12, 2001.

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Xiaochun Zhu

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Respectfully submitted,



Xiaochun Zhu

Registration No. 56,311

Scully, Scott, Murphy & Presser, P. C.
400 Garden City Plaza-STE 300
Garden City, New York 11530
Telephone: 516-742-4343

XZ:ab
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I, MICHELLE HENKEL, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PR 7618 for a patent by THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH as filed on 12 September 2001.

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A U S T R A L I A

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"A Method of Diagnosis and Treatment and Agents Useful for Same"

The invention is described in the following statement:

- 1 -

A METHOD OF DIAGNOSIS AND TREATMENT AND AGENTS USEFUL FOR SAME

FIELD OF THE INVENTION

5

The present invention relates generally to a method for detecting an aberrant cell, and more particularly an aberrant breast cell, in a subject or in a biological sample from said subject and agents useful for same. The presence of the aberrant cell or group of aberrant cells provides an indication of a particular disease or condition or a propensity for development of a disease or condition. More particularly, the present invention contemplates a method for detecting a cell associated with breast cancer or having a propensity to develop into a breast cancer in a subject or in a biological sample from said subject by determining the relative increase in the presence of the LM04 protein or a related protein or a relative increase in LM04 activity or a relative increase in the presence of expression products from a gene encoding the LM04 protein or a related protein. The present invention further provides a method for diagnosing the presence of a cancer or cancerous-like growth, in particular breast cancer in a subject or in a biological sample from said subject by screening for up-regulation of LM04 or a related protein in a cell or group of cells or an up-regulation in the presence of expression products of genetic sequences encoding LM04 or a related protein. The present invention provides diagnostic agents useful for detecting LM04 or expression products of genetic material encoding LM04. Such diagnostic agents include immunointeractive molecules, such as antibodies, and genetic probes for detecting expression products of LM04 genes. The present invention further provides genetically modified animals exhibiting altered levels of LM04. Such animals are useful models for screening for anti-cancer agents.

The present invention still further relates generally to a method of modulating LM04 related cellular proliferation and to agents useful for same. More particularly, the present

- 2 -

invention contemplates a method of modulating breast cell proliferation by modulating LM04 nucleic acid expression and/or LM04 functioning. The method of the present invention is useful, *inter alia*, in the treatment and/or prophylaxis of conditions characterised by aberrant, unwanted or otherwise inappropriate LM04 regulated cellular proliferation. The present invention is further directed to methods for identifying and/or designing agents capable of modulating LM04 regulated cellular proliferation.

BACKGROUND OF THE INVENTION

- 10 Bibliographic details of the publications referred to by both author and numerically in this specification are collected at the end of the description.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that that prior art forms part of the common general knowledge in Australia.

15 The LIM domain defines a conserved cysteine-rich structure comprising two tandemly repeated zinc fingers and is found in a large group of diverse proteins (reviewed in 1, 2). This motif, originally identified in LIM-homeodomain transcription factors, may either occur alone (as one or more copies) or in association with heterologous domains such as a protein kinase or homeobox domain. Targeted gene disruption has established that LIM domain-containing proteins have critical functions in cell-fate specification and differentiation (3).

25 The LMO family consists of four members (designated LM01-4), each of which comprises two tandem LIM domains. LM02 is essential for embryonic hematopoiesis and is thought to function at the level of the pluripotent stem cell (11). Little is known about the function of LM03, discovered on the basis of sequence homology. The most

- 3 -

recently described member, LM04, was isolated by virtue of its interaction with Ldb1/NL1/CLIM and in an expression screen with autologous serum (12-15). Ldb1 is a multifunctional adaptor protein that interacts with LMO proteins and other nuclear LIM-containing factors (16-19). The LM04 gene is widely expressed in both embryonic and adult tissues (12, 13, 15). Like the other members of this family, it is presumed that LM04 is a transcriptional cofactor that primarily functions as a docking site for other factors. LM04 may also contribute an activation or repression domain to influence transcriptional activity.

- 10 In work leading up to the present invention the inventors have determined that LM04 plays a role in mammary development and in breast oncogenesis. The LM04 gene is developmentally regulated in mammary epithelium and LM04 together with Ldb1 have been found to act as negative regulators of mammary epithelial differentiation. Significantly, overexpression of the LM04 gene was found in greater than 50% of primary breast cancers, indicating that this protein contributes to the pathogenesis of breast cancer.
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SUMMARY OF THE INVENTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising",
5 will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

One aspect of the present invention contemplates a method for detecting an aberrant cell in a subject or in a biological sample from said subject, said method comprising
10 contacting cells or cell extracts from said subject or said biological sample with an immunointeractive molecule specific for LM04 or antigenic portion thereof and screening for the level of immunointeractive molecule-LM04 complex formation wherein an elevated presence of said complex relative to a normal cell is indicative of an aberrant cell.

15 Another aspect of the present invention contemplates a method for detecting an aberrant mammary cell in a subject or in a biological sample from said subject, said method comprising contacting mammary cells or mammary cell extracts from said subject or said biological sample with an immunointeractive molecule specific for LM04 or antigenic
20 portion thereof and screening for the level of immunointeractive molecule-LM04 complex formation wherein an elevated presence of said complex relative to a normal breast cell is indicative of an aberrant mammary cell.

25 In a related aspect, the present invention provides a method for detecting an aberrant cell in a subject or in a biological sample from said subject, said method comprising screening the level of an expression product of a gene encoding LM04 wherein an elevated level of said expression product compared to a normal cell is indicative of an aberrant cell.

- 5 -

In another aspect there is provided a method for detecting an aberrant mammary cell in a subject or in a biological sample from said subject, said method comprising screening the level of an expression product of a gene encoding LM04 wherein an elevated level of said expression product compared to a normal mammary cell is indicative of an aberrant mammary cell.

In yet another aspect of the present invention contemplates a method for diagnosing the presence of a neoplasm or neoplasm-like growth in a subject, said method comprising contacting cells or cell extracts from said subject or a biological sample from said subject with a LM04-binding effective amount of an antibody having specificity for said LM04 or an antigenic determinant or epitope thereon and then quantitatively or qualitatively determining the level of a LM04-antibody complex wherein the presence of elevated levels of said complex compared to a normal cell is indicative of the presence of a neoplasm.

In a related aspect the present invention provides a method for diagnosing the presence of a neoplasm in a subject, said method comprising obtaining mRNA from cells of said subject or from a biological sample from said subject and optionally generating cDNA and contacting said mRNA or cDNA with a genetic probe capable of hybridizing to and/or amplifying all or part of a nucleotide sequence encoding LM04 or its complementary nucleotide sequence and then detecting the level of said mRNA or cDNA wherein the presence of elevated levels of said mRNA or cDNA compared to normal controls is indicative of the presence of a neoplasm.

In still another aspect the present invention contemplates a deimmunized antibody molecule having specificity for an epitope recognized by a monoclonal antibody to LM04 wherein at least one of the CDRs of the variable domain of said deimmunized antibody is derived from the said monoclonal antibody to LM04 and the remaining immunoglobulin-

- 6 -

derived parts of the deimmunized antibody molecule are derived from an immunoglobulin or an analogue thereof from the host for which the antibody is to be deimmunized.

5 The present invention further contemplates an assay to detect LM04 including the steps of:-

- (1) contacting a monoclonal antibody specific to LM04 or an antigenic determinant thereon with a biological sample suspected of containing a cell containing said
10 LM04; and
- (2) subjecting the complex formed in step (1) to a signal detection step.

Another aspect of the present invention contemplates a method for detecting neoplastic
15 cells in a human patient, said method comprising introducing into said patient a deimmunized form of a non-human derived monoclonal antibody specific for human LM04 or an antigenic determinant thereon labelled with a reporter molecule, allowing dissemination of the labelled antibody throughout the circulatory system, or to selected parts of the circulatory system and then subjecting said patient to reporter molecule-
20 detection means to identify the location of the antibody.

Yet another aspect of the present invention provides a method of detecting, in a sample, LM04 or fragment, variant or derivative thereof comprising contacting the sample with an antibody or fragment or derivative thereof and detecting the level of a complex
25 comprising said antibody and LM04 or fragment, variant or derivative thereof compared to normal controls wherein elevated levels of LM04 is indicative of cancer growth.

Accordingly, one aspect of the present invention provides a method of modulating LM04 regulated cellular proliferation, said method comprising contacting said cell with an

- 7 -

effective amount of an agent for a time and under conditions sufficient to modulate *LM04* expression or *LM04* functional activity wherein inhibiting or otherwise antagonising said expression or activity down-regulates said cellular proliferation.

- 5 In a further aspect the present invention provides a method of modulating *LM04*-regulated mammary cell proliferation, said method comprising contacting said cell with an effective amount of an agent for a time and under conditions sufficient to modulate *LM04* expression or *LM04* functional activity wherein inhibiting or otherwise antagonising said expression or activity down-regulates said cellular proliferation.

10

In yet another aspect, the present invention is directed to the method for the treatment and/or prophylaxis of a conditions characterised by aberrant, unwanted or otherwise inappropriate *LM04*-regulated proliferative activity in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and
15 under conditions sufficient to modulate *LM04* expression or *LM04* functional activity wherein inhibiting or otherwise antagonising said expression or activity down-regulates said cellular proliferation.

- 20 In still another aspect there is provided a method for the treatment and/or prophylaxis of a neoplastic condition said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to down-regulate *LM04* expression or *LM04* functional activity wherein inhibiting or otherwise antagonising said expression or activity down-regulates cellular proliferation.

- 25 Another aspect of the present invention contemplates the use of an agent, as hereinbefore defined, in the manufacture of medicament for the treatment of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate *LM04*-regulated cellular proliferation, wherein said agent modulates *LM04* expression or *LM04*

- 8 -

activity and wherein inhibiting or otherwise antagonising said expression or activity down-regulates said cellular proliferation.

5 In yet another further aspect, the present invention contemplates a pharmaceutical composition comprising the modulatory agent as hereinbefore defined together with one or more pharmaceutically acceptable carriers and/or diluents. Said agents are referred to as the active ingredients.

10 In another aspect the present invention provides a method for detecting an agent capable of modulating *LM04* expression or LM04 functional activity said method comprising contacting a cell or extract thereof containing said *LM04* or LM04 with a putative agent and detecting an altered expression phenotype associated with said interaction.

15 In a related aspect, the present invention should be understood to extend to the agents identified utilising any of the methods hereinbefore defined. In this regard, reference to an agent should be understood as a reference to any proteinaceous or non-proteinaceous molecule which modulates at least one LM04-related functional activity.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 LM04 is abundantly expressed in the lobular-alveolar units during pregnancy.

(A) A single layer of ductal epithelium expressing LM04 transcripts is evident in the adult mammary gland. RNA expression was assessed by *in situ* hybridisation using sense (control) and antisense digoxigenin-labeled riboprobes corresponding to full-length mouse LM04 sequence. Orig. mag. 50x (B) Northern analysis of mammary glands from virgin, pregnant, lactating and force-weaned (involution) mice. Filters containing 20 µg total RNA were hybridised with a mouse LM04 probe. The lower molecular weight bands may represent either an alternatively spliced variant of LM04 RNA or a cross-hybridising species. The ethidium bromide stained gel showing 28S and 18S ribosomal RNA provides a loading control.

Figure 2 LM04 and Ldb1 inhibit β -casein synthesis in SCp2 mammary cells induced to

differentiate. (A) RT-PCR analysis was performed using total RNA derived from stably transfected SCp2 pools that were stimulated (+) with prolactin, insulin and hydrocortisone or unstimulated (-) for 96 hrs. β -casein and HPRT were used as markers of differentiation and loading, respectively. At least five independent transfections were performed. PCR products were fractionated by gel electrophoresis, blotted and hybridised with internal oligonucleotide probes. The ethidium bromide stained gel for Flag-Ldb1 is shown in rightmost panel. (B) Immunoprecipitation and Western blot analysis confirmed expression of Flag-LM04 and Flag-Ldb1 in SCp2 transfectants. Lysates from cells expressing either gene or containing empty vector were subjected to immunoprecipitation using mouse anti-Flag antibody and then blotted with rabbit anti-LM04 or rabbit anti-Ldb1 antibody. Arrows indicate relevant proteins.

Figure 3 LM04 is overexpressed in several human breast cancer cell lines. Northern analysis of poly(A)⁺ RNA (3 µg) from human and mouse (SCp2) breast epithelial cell

lines. Filters were sequentially hybridised with mouse LM04, Ldb1 and GAPDH cDNA probes. The lower molecular weight Ldb1 transcripts in human breast cancer cell lines may represent a cross-hybridising species. Size of the Ldb1 transcripts appeared to be lower in the mouse SCp2 cell line relative to those in human cell lines.

5

Figure 4 Overexpression of LM04 in primary breast cancer. In situ hybridisation was performed on tissue arrays containing archival breast specimens, using full-length human LM04 sense and antisense riboprobes labeled with digoxigenin (A-E). High LM04 expression in infiltrating ductal (A, C) and lobular (B) carcinomas (antisense probe). (D) Normal lobule, displaying low expression of LM04. (E) Ductal carcinoma *in situ* (DCIS), showing high expression of LM04. Inserts in (B) and (C) represent tumors hybridised with the sense (control) riboprobe. Immunohistochemistry was performed on tissue arrays using a rat anti-LM04 monoclonal antibody (F-H). (F) Abundant protein expression detected in the corresponding infiltrating lobular cancer sample as for (B). (G) Protein staining for the same infiltrating ductal carcinoma as shown in (C). (H) High level protein expression in an infiltrating ductal carcinoma. Negative controls for immunostaining are shown in the inserts within (F) and (G). Orig. mag. 44x.

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Figure 5 Interaction between LM04 and CtIP requires a single LIM domain and appears to be specific for the LMO subclass. (a) CtIP associates with LMO4, LMO2 and a single LIM domain of LMO4. Cell extracts from transiently transfected 293T cells were coimmunoprecipitated with the indicated specific antisera, then immunoblotted using the antibody shown under each panel. F, Flag; C, control isotype-matched monoclonal antibody. Western blot analysis confirmed expression of CtIP and LMO proteins. (b) Heterologous LIM proteins do not interact with CtIP. Cell extracts were analysed as in (a). (c) Interaction between endogenous LMO4 and CtIP proteins in breast epithelial cells. Nuclear extracts were analysed as in (a). Western blot analysis of nuclear extract loaded in an adjacent lane provided a size control for CtIP.

Figure 6 Coexpression of LMO4 and CtIP in breast epithelial cells. (a) Northern blot analysis of poly(A)⁺ RNA isolated from the indicated 'normal' and human breast cancer cell lines. The filter was sequentially hybridized with human CtIP and LMO4 cDNA probes, followed by the control GAPDH probe. (b) Nuclear and cytoplasmic localization of endogenous LMO4 in breast epithelial cells. T-47D cells were stained with rat α -LMO4 antibody or control monoclonal antibody, and analysed by indirect immunofluorescence.

Figure 7 Two regions within CtIP specifically interact with LMO4. (a) Schematic representation of wild-type CtIP and deletion mutant constructs that were tested for interaction with LMO4 in mammalian cells. The region that associates with BRCA1 and two putative leucine zipper (LZ) domains are indicated. A summary of each mutant's ability to interact with LMO4 is shown. (b) Cell extracts were coimmunoprecipitated with α -Flag or isotype-matched monoclonal antibody, then blotted with α -HA antibody, specific for CtIP protein. Western blot analysis confirmed expression of Flag-LMO4 and CtIP mutants (lower panels).

Figure 8 LMO4 forms a complex with CtIP and BRCA1 *in vivo* and directly interacts with BRCA1. (a) Specific interaction between LMO4, CtIP and BRCA1 *in vivo*. Cell extracts were immunoprecipitated with α -myc antibody then blotted with either α -Flag or α -CtIP antibodies. Expression of individual proteins in transfected 293T cell extracts is shown below. (b) Endogenous LMO4 and BRCA1 proteins associate in breast epithelial cells. Nuclear extracts were immunoprecipitated with rat α -LMO4 monoclonal or control antibody, then immunoblotted with α -BRCA1 monoclonal antibody. (c) Interaction between LMO4, CtIP, BRCA1 and Ldb1 *in vivo*. Cell extracts were immunoprecipitated with anti-CtIP antibody, then immunoblotted with the indicated antibodies. Western blot analysis confirmed the expression of individual proteins in the transfected cells.

- 12 -

Figure 9 The C-terminal BRCT domains of BRCA1 mediate interaction with LMO4. (a) Cell lysates were analysed with the indicated antibodies, as above. (b) LMO4 and the BRCT domain interact *in vitro*. *In vitro* translated ³⁵S-methionine-labeled BRCA1 (aa 1528 – 1863), corresponding to the SZ fragment, was incubated with GST-LMO4 fusion protein immobilised on glutathione sepharose. Bound proteins were analysed by SDS-PAGE. Arrow depicts the BRCA1-SZ protein.

Figure 10 LMO4 represses BRCA1 activity in both yeast and mammalian transcriptional activation assays. (a) Activation of β -galactosidase expression in BJ5462 cells expressing either GAL4-DBD (DNA-binding domain) or GAL4-BRCA1-AD (activation domain) fusion protein in the presence or absence of a yeast LMO4 expression vector. (b) Activity of the BRCA1 activation domain in 293T cells cotransfected with the pG5CAT reporter plasmid, containing five Gal binding sites upstream of the chloramphenicol acetyltransferase gene, and either an LMO4 expression construct or empty vector. The BRCA1-AD domain was fused to the GAL4-DBD. Basal activity of the GAL4-DBD is shown in both yeast and mammalian cells. The data in A and B represent an average of three independent experiments with standard error of the mean indicated. Western blot analysis confirmed expression of the relevant proteins, designated by arrows, in yeast or mammalian cells.

Figure 11 A potential role for LMO4 as a repressor of BRCA1 activity in breast epithelial cells. Overexpression of LMO4 in breast cancer cells may attenuate BRCA1-mediated activation of certain genes. BRCA1 may achieve this by recruitment of transcription factors or by altering accessibility of proteins to a specific region by chromatin remodeling. BRCA1 appears to function in a large multiprotein complex, illustrated by the presence of proteins X, Y and Z.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is predicated in part on the determination that LM04 expression is regulated in cancer cells and in particular in breast cancer cells relative to normal breast cancer cells. The identification of this cancer-specific marker permits development of a range of diagnostic agents, including cancer imaging agents and cancer targeting agents having therapeutic applications. These determinations also permit the rational design of therapeutic and/or prophylactic methods for treating conditions, such as those characterised by aberrant or unwanted LM04 regulated proliferation.

Accordingly, one aspect of the present invention contemplates a method for detecting an aberrant cell in a subject or in a biological sample from said subject, said method comprising contacting cells or cell extracts from said subject or said biological sample with an immunointeractive molecule specific for LM04 or antigenic portion thereof and screening for the level of immunointeractive molecule-LM04 complex formation wherein an elevated presence of said complex relative to a normal cell is indicative of an aberrant cell.

More particularly, the present invention contemplates a method for detecting an aberrant mammary cell in a subject or in a biological sample from said subject, said method comprising contacting mammary cells or mammary cell extracts from said subject or said biological sample with an immunointeractive molecule specific for LM04 or antigenic portion thereof and screening for the level of immunointeractive molecule-LM04 complex formation wherein an elevated presence of said complex relative to a normal breast cell is indicative of an aberrant mammary cell.

In a related embodiment, the present invention provides a method for detecting an aberrant cell in a subject or in a biological sample from said subject, said method

- 14 -

comprising screening the level of an expression product of a gene encoding LM04 wherein an elevated level of said expression product compared to a normal cell is indicative of an aberrant cell.

5 More particularly, there is provided a method for detecting an aberrant mammary cell in a subject or in a biological sample from said subject, said method comprising screening the level of an expression product of a gene encoding LM04 wherein an elevated level of said expression product compared to a normal mammary cell is indicative of an aberrant mammary cell.

10

Reference herein to a "subject" includes an animal including a mammal such as a human, primate, laboratory test animal (e.g. mouse, rabbit, rat, guinea pig), livestock animal (e.g. sheep, cow, pig, horse) or companion animal (e.g. cat, dog). The preferred subject is a human.

15

Reference herein to "mammary" cells or tissues should be understood as a reference to the tissues comprising and associated with the milk producing glands. This term should be understood to encompass mammary tissues and cells, irrespective of whether the mammary gland is actually lactating. Such tissue includes but is not limited to, mammary epithelial cells, stromal cell, the lactiferous duct, ampulla, glandular tissue, areola and nipple. The mammary gland is often referred to by alternative terms such as the "breast" in human. The terms "mammary" and "breast" are herein utilised interchangeably.

20

25 A "biological sample" from a subject includes a biopsy and may be any sample of cells, cell extract, tissue, tissue fluid, excretia, circulatory fluid or respiratory fluid or other material such as fluid or other material which is introduced into a subject and subsequently withdrawn for analysis. The biological sample may be extracted, treated,

- 15 -

untreated, diluted or concentrated from the subject.

Reference to a "normal" cell includes a cell not regarded as aberrant or cancerous and may be considered an "average" of normal cell types.

5

The "immunointeractive molecule" is any molecule having specificity and binding affinity for LM04 or its antigenic parts or its homologues or derivatives. Although the preferred immunointeractive molecule is an immunoglobulin molecule, the present invention extends to other immunointeractive molecules such as antibody fragments,
10 single chain antibodies, deimmunized including humanized antibodies and T-cell associated antigen-binding molecules (TABMs). Most preferably, the immunointeractive molecule is an antibody such as a polyclonal or monoclonal antibody. Most preferably, the antibody is a monoclonal antibody.

15

The immunointeractive molecule exhibits specificity for LM04 or more particularly an antigenic determinant or epitope on LM04. An antigenic determinant or epitope on LM04 includes that part of the molecule to which an immune response is directed. The antigenic determinant or epitope may be a B-cell epitope or where appropriate a T-cell epitope. The term "antigenic part" includes an antigenic determinant or epitope.

20

An "expression product" is generally mRNA or cDNA and the amount of expression product provides an indicator of the level of LM04 gene expression and provides, therefore, indirect evidence for the presence of LM04. Conveniently, pools of mRNA or cDNA are obtained or cell extracts comprising total mRNA obtained and genetic probes
25 complementary to all or part of the LM04 gene-specific mRNA or cDNA. Binding of probes may then be quantitated or semi-quantitated.

Reference herein to LM04 includes reference to all forms of LM04 or their homologues

- 16 -

or derivatives. Reference to "LM04" should be understood to include reference to any isoforms which arise from alternative splicing of LM04 mRNA or mutants or polymorphic variants of LM04. It should also be understood to include reference to any other molecule which exhibits LM04 functional activity to the extent that the subject molecule mimics one or more LM04 cellular proliferation related activities. It is conceivable, for example, that there may be naturally or non-naturally occurring LM04 mimetics (for example, toxins or drugs) which, if they were introduced into an individual, would induce unwanted cellular proliferation. Reference to "*LM04*" in italicised text should be understood as a reference to LM04 encoding nucleic acid molecules while "LM04" in not italicised text is a reference to the LM04 protein.

Reference to a "level" of LM04 includes an amount quantitatively, semi-quantitatively or qualitatively determined.

In accordance with the present invention, it is proposed that cells associated with a cancer including cancer cells produce elevated levels of LM04. The quantitative or qualitative detection of levels of LM04 or expression products of genetic material encoding LM04 provides, therefore, an indicator that a cell is aberrant and is associated with cancer or has a propensity to develop into a cancer.

Accordingly, another aspect of the present invention contemplates a method for diagnosing the presence of a neoplasm or neoplasm-like growth in a subject, said method comprising contacting cells or cell extracts from said subject or a biological sample from said subject with a LM04-binding effective amount of an antibody having specificity for said LM04 or an antigenic determinant or epitope thereon and then quantitatively or qualitatively determining the level of a LM04-antibody complex wherein the presence of elevated levels of said complex compared to a normal cell is indicative of the presence of a neoplasm.

- 17 -

More particularly, said neoplasm is breast cancer.

In a related embodiment, the present invention provides a method for diagnosing the presence of a neoplasm in a subject, said method comprising obtaining mRNA from cells
5 of said subject or from a biological sample from said subject and optionally generating cDNA and contacting said mRNA or cDNA with a genetic probe capable of hybridizing to and/or amplifying all or part of a nucleotide sequence encoding LM04 or its complementary nucleotide sequence and then detecting the level of said mRNA or cDNA wherein the presence of elevated levels of said mRNA or cDNA compared to normal
10 controls is indicative of the presence of a neoplasm.

More particularly, said neoplasm is breast cancer.

Reference to a "neoplasm" should be understood as a reference to a lesion, tumour or
15 other encapsulated or unencapsulated mass or other form of growth which comprises neoplastic cells. A "neoplastic cell" should be understood as a reference to a cell exhibiting abnormal growth. The term "growth" should be understood in its broadest sense and includes reference to proliferation. In this regard, an example of abnormal cell growth is the uncontrolled proliferation of a cell. The neoplastic cell may be a benign
20 cell or a malignant cell. In a preferred embodiment, the subject neoplasm is a breast cancer.

The use of antibodies and in particular monoclonal antibodies to detect LM04 is a preferred method of the present invention. Antibodies may be prepared by any of a
25 number of means. For the detection of human LM04, antibodies are generally but not necessarily derived from non-human animals such as primates, livestock animals (e.g. sheep, cows, pigs, goats, horses), laboratory test animals (e.g. mice, rats, guinea pigs, rabbits) and companion animals (e.g. dogs, cats). Generally, antibody based assays are

conducted *in vitro* on cell or tissue biopsies. However, if an antibody is suitably deimmunized or, in the case of human use, humanized, then the antibody can be labelled with, for example, a nuclear tag, administered to a patient and the site of nuclear label accumulation determined by radiological techniques. The LM04 antibody is regarded, therefore, as a cancer targeting agent. Accordingly, the present invention extends to deimmunized forms of the antibodies for use in cancer imaging in human and non-human patients. This is described further below.

The present invention provides, therefore, an antibody and in particular a monoclonal antibody for use in immunological assays for LM04 or for cancer imaging *in vivo*. The antibody may be directed either to the LM04 protein or the LM04 mRNA, for example.

For the generation of antibodies to a LM04, this molecule is required to be extracted from a biological sample whether this be from animal including human tissue or from cell culture if produced by recombinant means. The LM04 can be separated from the biological sample by any suitable means. For example, the separation may take advantage of any one or more of LM04's surface charge properties, size, density, biological activity and its affinity for another entity (e.g. another protein or chemical compound to which it binds or otherwise associates). Thus, for example, separation of LM04 from the biological fluid may be achieved by any one or more of ultra-centrifugation, ion-exchange chromatography (e.g. anion exchange chromatography, cation exchange chromatography), electrophoresis (e.g. polyacrylamide gel electrophoresis, isoelectric focussing), size separation (e.g., gel filtration, ultra-filtration) and affinity-mediated separation (e.g. immunoaffinity separation including, but not limited to, magnetic bead separation such as Dynabead™ separation, immunochromatography, immuno-precipitation). Choice of the separation technique(s) employed may depend on the biological activity or physical properties of the LM04 sought or from which tissues it is obtained.

Preferably, the separation of LM04 from the biological fluid preserves conformational epitopes present on the protein and, thus, suitably avoids techniques that cause denaturation of the enzyme. Persons of skill in the art will recognize the importance of maintaining or mimicking as close as possible physiological conditions peculiar to the LM04 (e.g. the biological fluid from which it is obtained) to ensure that the antigenic determinants or active site/s on the LM04, which are exposed to the animal, are structurally identical to that of the native protein. This ensures the raising of appropriate antibodies in the immunised animal that would recognize the native protein. In a preferred embodiment, LM04 is separated from the biological fluid using any one or more of affinity separation, gel filtration and ultra-filtration.

Immunization and subsequent production of monoclonal antibodies can be carried out using standard protocols as for example described by Köhler and Milstein (1975, 1976), Coligan *et al.* (1991-1997) or Toyama *et al.* (1987). Essentially, an animal is immunized with a LM04-containing biological fluid or fraction thereof by standard methods to produce antibody-producing cells, particularly antibody-producing somatic cells (e.g. B lymphocytes). These cells can then be removed from the immunized animal for immortalization.

Where a fragment of LM04 is used to generate antibodies, it may need to first be associated with a carrier. By "carrier" is meant any substance of typically high molecular weight to which a non- or poorly immunogenic substance (e.g. a hapten) is naturally or artificially linked to enhance its immunogenicity.

Immortalization of antibody-producing cells may be carried out using methods which are well-known in the art. For example, the immortalization may be achieved by the transformation method using Epstein-Barr virus (EBV) (Kozbor *et al.*, 1986). In a preferred embodiment, antibody-producing cells are immortalized using the cell fusion

- 20 -

method (described in (Coligan *et al.*, 1991-1997)), which is widely employed for the production of monoclonal antibodies. In this method, somatic antibody-producing cells with the potential to produce antibodies, particularly B cells, are fused with a myeloma cell line. These somatic cells may be derived from the lymph nodes, spleens and peripheral blood of primed animals, preferably rodent animals such as mice and rats. Mice spleen cells are particularly useful. It would be possible, however, to use rat, rabbit, sheep or goat cells, or cells from other animal species instead.

Specialized myeloma cell lines have been developed from lymphocytic tumours for use in hybridoma-producing fusion procedures (Kohler and Milstein, 1976; Shulman *et al.*, 1978; Volk *et al.*, 1982). These cell lines have been developed for at least three reasons. The first is to facilitate the selection of fused hybridomas from unfused and similarly indefinitely self-propagating myeloma cells. Usually, this is accomplished by using myelomas with enzyme deficiencies that render them incapable of growing in certain selective media that support the growth of hybridomas. The second reason arises from the inherent ability of lymphocytic tumour cells to produce their own antibodies. To eliminate the production of tumour cell antibodies by the hybridomas, myeloma cell lines incapable of producing endogenous light or heavy immunoglobulin chains are used. A third reason for selection of these cell lines is for their suitability and efficiency for fusion.

Many myeloma cell lines may be used for the production of fused cell hybrids, including, e.g. P3X63-Ag8, P3X63-AG8.653, P3/NS1-Ag4-1 (NS-1), Sp2/0-Ag14 and S194/5.XXO.Bu.1. The P3X63-Ag8 and NS-1 cell lines have been described by Köhler and Milstein (1976). Shulman *et al.* (1978) developed the Sp2/0-Ag14 myeloma line. The S194/5.XXO.Bu.1 line was reported by Trowbridge (1978).

- 21 -

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually involve mixing somatic cells with myeloma cells in a 10:1 proportion (although the proportion may vary from about 20:1 to about 1:1), respectively, in the presence of an agent or agents (chemical, viral or electrical) that promotes the fusion of cell membranes. Fusion methods have been described (Kohler and Milstein, 1975; 1976; Gafter *et al.*, 1977; Volk *et al.*, 1982). The fusion-promoting agents used by those investigators were Sendai virus and polyethylene glycol (PEG).

Because fusion procedures produce viable hybrids at very low frequency (e.g. when spleens are used as a source of somatic cells, only one hybrid is obtained for roughly every 1×10^5 spleen cells), it is preferable to have a means of selecting the fused cell hybrids from the remaining unfused cells, particularly the unfused myeloma cells. A means of detecting the desired antibody-producing hybridomas among other resulting fused cell hybrids is also necessary. Generally, the selection of fused cell hybrids is accomplished by culturing the cells in media that support the growth of hybridomas but prevent the growth of the unfused myeloma cells, which normally would go on dividing indefinitely. The somatic cells used in the fusion do not maintain long-term viability in *in vitro* culture and hence do not pose a problem. In the example of the present invention, myeloma cells lacking hypoxanthine phosphoribosyl transferase (HPRT-negative) were used. Selection against these cells is made in hypoxanthine/aminopterin/thymidine (HAT) medium, a medium in which the fused cell hybrids survive due to the HPRT-positive genotype of the spleen cells. The use of myeloma cells with different genetic deficiencies (drug sensitivities, etc.) that can be selected against in media supporting the growth of genotypically competent hybrids is also possible.

Several weeks are required to selectively culture the fused cell hybrids. Early in this time period, it is necessary to identify those hybrids which produce the desired antibody, so that they may subsequently be cloned and propagated. Generally, around 10% of the

hybrids obtained produce the desired antibody, although a range of from about 1 to about 30% is not uncommon. The detection of antibody-producing hybrids can be achieved by any one of several standard assay methods, including enzyme-linked immunoassay and radioimmunoassay techniques as, for example, described in Kennet *et al.* (1980) and by FACS analysis (1998).

Once the desired fused cell hybrids have been selected and cloned into individual antibody-producing cell lines, each cell line may be propagated in either of two standard ways. A suspension of the hybridoma cells can be injected into a histocompatible animal.

The injected animal will then develop tumours that secrete the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can be tapped to provide monoclonal antibodies in high concentration. Alternatively, the individual cell lines may be propagated *in vitro* in laboratory culture vessels. The culture medium containing high concentrations of a single specific monoclonal antibody can be harvested by decantation, filtration or centrifugation, and subsequently purified.

The cell lines are tested for their specificity to detect the LM04 by any suitable immunodetection means. For example, cell lines can be aliquoted into a number of wells and incubated and the supernatant from each well is analyzed by enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody technique, or the like. The cell line(s) producing a monoclonal antibody capable of recognizing the target LM04 but which does not recognize non-target epitopes are identified and then directly cultured *in vitro* or injected into a histocompatible animal to form tumours and to produce, collect and purify the required antibodies.

These antibodies are LM04 specific. This means that the antibodies are capable of distinguishing LM04 from other molecules. More broad spectrum antibodies may be used

provided that they do not cross react with molecules in a normal cell.

Where the monoclonal antibody is destined for use in *in vivo* cancer imaging or treatment, it will need to be deimmunized with respect to the host into which it will be introduced (e.g. a human). The deimmunization process may take any of a number of forms including the preparation of chimeric antibodies which have the same or similar specificity as the monoclonal antibodies prepared according to the present invention. Chimeric antibodies are antibodies whose light and heavy chain genes have been constructed, typically by genetic engineering, from immunoglobulin variable and constant region genes belonging to different species. Thus, in accordance with the present invention, once a hybridoma producing the desired monoclonal antibody is obtained, techniques are used to produce interspecific monoclonal antibodies wherein the binding region of one species is combined with a non-binding region of the antibody of another species (Liu *et al.*, 1987). For example, complementary determining regions (CDRs) from a non-human (e.g. murine) monoclonal antibody can be grafted onto a human antibody, thereby "humanizing" the murine antibody (European Patent Publication No. 0 239 400; Jones *et al.*, 1986; Verhoeyen *et al.*, 1988; Richmann *et al.*, 1988). In this case, the deimmunizing process is specific for humans. More particularly, the CDRs can be grafted onto a human antibody variable region with or without human constant regions. The non-human antibody providing the CDRs is typically referred to as the "donor" and the human antibody providing the framework is typically referred to as the "acceptor". Constant regions need not be present, but if they are, they must be substantially identical to human immunoglobulin constant regions, i.e. at least about 85-90%, preferably about 95% or more identical. Hence, all parts of a humanized antibody, except possibly the CDRs, are substantially identical to corresponding parts of natural human immunoglobulin sequences. Thus, a "humanized antibody" is an antibody comprising a humanized light chain and a humanized heavy chain immunoglobulin. A donor antibody is said to be "humanized", by the process of "humanization", because the resultant

- 24 -

humanized antibody is expected to bind to the same antigen as the donor antibody that provides the CDRs. Reference herein to "humanized" includes reference to an antibody deimmunized to a particular host, in this case, a human host.

- 5 It will be understood that the deimmunized antibodies may have additional conservative amino acid substitutions which have substantially no effect on antigen binding or other immunoglobulin functions. Exemplary conservative substitutions may be made according to Table 1.

TABLE 1

ORIGINAL RESIDUE	EXEMPLARY SUBSTITUTIONS
Ala	Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Pro
His	Asn, Gln
Ile	Leu, Val
Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Ile
Phe	Met, Leu, Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp, Phe
Val	Ile, Leu

Exemplary methods which may be employed to produce deimmunized antibodies according to the present invention are described, for example, in references Richmann *et al.*, 1988; European Patent Publication No. 0 239 400; Chou *et al.*; Queen *et al.*; Morgan *et al.*

- 26 -

Thus, in one embodiment, the present invention contemplates a deimmunized antibody molecule having specificity for an epitope recognized by a monoclonal antibody to LM04 wherein at least one of the CDRs of the variable domain of said deimmunized antibody is derived from the said monoclonal antibody to LM04 and the remaining immunoglobulin-derived parts of the deimmunized antibody molecule are derived from an immunoglobulin or an analogue thereof from the host for which the antibody is to be deimmunized.

This aspect of the present invention involves manipulation of the framework region of a non-human antibody.

The present invention extends to mutants and derivatives of the subject antibodies but which still retain specificity for LM04.

The terms "mutant" or "derivatives" includes one or more amino acid substitutions, additions and/or deletions.

As used herein, the term "CDR" includes CDR structural loops which covers to the three light chain and the three heavy chain regions in the variable portion of an antibody framework region which bridge β strands on the binding portion of the molecule. These loops have characteristic canonical structures (Chothia *et al.*, 1987; Chothia *et al.*, 1992).

By "framework region" is meant region of an immunoglobulin light or heavy chain variable region, which is interrupted by three hypervariable regions, also called CDRs.

The extent of the framework region and CDRs have been precisely defined (see, for example, Kabat *et al.*, 1983). The sequences of the framework regions of different light or heavy chains are relatively conserved within a species. As used herein, a "human framework region" is a framework region that is substantially identical (about 85% or

more, usually 90-95% or more) to the framework region of a naturally occurring human immunoglobulin. The framework region of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDRs. The CDRs are primarily responsible for binding to an epitope of LM04.

5

As used herein, the term "heavy chain variable region" means a polypeptide which is from about 110 to 125 amino acid residues in length, the amino acid sequence of which corresponds to that of a heavy chain of a monoclonal antibody of the invention, starting from the amino-terminal (N-terminal) amino acid residue of the heavy chain.

10 Likewise, the term "light chain variable region" means a polypeptide which is from about 95 to 130 amino acid residues in length, the amino acid sequence of which corresponds to that of a light chain of a monoclonal antibody of the invention, starting from the N-terminal amino acid residue of the light chain. Full-length immunoglobulin "light chains" (about 25 Kd or 214 amino acids) are encoded by a variable region gene at the NH₂-terminus (about 110 amino acids) and a κ or λ constant region gene at the COOH-terminus. Full-length immunoglobulin "heavy chains" (about 50 Kd or 446 amino acids), are similarly encoded by a variable region gene (about 116 amino acids) and one of the other aforementioned constant region genes, e.g. γ (encoding about 330 amino acids).

20 The term "immunoglobulin" or "antibody" is used herein to refer to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes. The recognized immunoglobulin genes include the κ , λ , α , γ (IgG₁, IgG₂, IgG₃, IgG₄), δ , ϵ and μ constant region genes, as well as the myriad immunoglobulin variable region genes. One form of immunoglobulin constitutes the basic structural unit of an antibody. This form is a tetramer and consists of two identical pairs of immunoglobulin chains, each pair having one light and one heavy chain. In each pair, the light and heavy chain variable regions are together responsible for binding to an antigen, and the constant regions are responsible for the antibody effector functions. In addition to antibodies,

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- 28 -

immunoglobulins may exist in a variety of other forms including, for example, Fv, Fab, Fab' and (Fab')₂.

5 The invention also contemplates the use and generation of fragments of monoclonal antibodies produced by the method of the present invention including, for example, Fv, Fab, Fab' and F(ab')₂ fragments. Such fragments may be prepared by standard methods as for example described by Coligan *et al.* (1991-1997).

10 The present invention also contemplates synthetic or recombinant antigen-binding molecules with the same or similar specificity as the monoclonal antibodies of the invention. Antigen-binding molecules of this type may comprise a synthetic stabilised Fv fragment. Exemplary fragments of this type include single chain Fv fragments (sFv, frequently termed scFv) in which a peptide linker is used to bridge the N terminus or C terminus of a V_H domain with the C terminus or N-terminus, respectively, of a V_L domain. ScFv lack all constant parts of whole antibodies and are not able to activate complement. Suitable peptide linkers for joining the V_H and V_L domains are those which allow the V_H and V_L domains to fold into a single polypeptide chain having an antigen binding site with a three dimensional structure similar to that of the antigen binding site of a whole antibody from which the Fv fragment is derived. Linkers having the desired properties may be obtained by the method disclosed in U.S. Patent No 4,946,778. However, in some cases a linker is absent. ScFvs may be prepared, for example, in accordance with methods outlined in Krebber *et al.* (Krebber *et al.*, 1997). Alternatively, they may be prepared by methods described in U.S. Patent No 5,091,513, European Patent No 239,400 or the articles by Winter and Milstein (Winter and Milstein, 1991) and Plückthun *et al.* (Plückthun *et al.*, 1996).

Alternatively, the synthetic stabilized Fv fragment comprises a disulphide stabilized Fv (dsFv) in which cysteine residues are introduced into the V_H and V_L domains such that in

- 29 -

the fully folded Fv molecule the two residues will form a disulphide bond therebetween. Suitable methods of producing dsFv are described, for example, in (Glockshuber *et al.*, 1990; Reiter *et al.*, 1994; Reiter *et al.*, 1994; Reiter *et al.*, 1994; Webber *et al.*, 1995).

5 Also contemplated as synthetic or recombinant antigen-binding molecules are single variable region domains (termed dAbs) as, for example, disclosed in (Ward *et al.*, 1989; Hamers-Casterman *et al.*, 1993; Davies & Reichmann, 1994).

10 Alternatively, the synthetic or recombinant antigen-binding molecule may comprise a "minibody". In this regard, minibodies are small versions of whole antibodies, which encode in a single chain the essential elements of a whole antibody. Suitably, the minibody is comprised of the V_H and V_L domains of a native antibody fused to the hinge region and CH3 domain of the immunoglobulin molecule as, for example, disclosed in U.S. Patent No 5,837,821.

15 In an alternate embodiment, the synthetic or recombinant antigen binding molecule may comprise non-immunoglobulin derived, protein frameworks. For example, reference may be made to (Ku & Schutz, 1995) which discloses a four-helix bundle protein cytochrome b562 having two loops randomized to create CDRs, which have been selected for antigen
20 binding.

The synthetic or recombinant antigen-binding molecule may be multivalent (i.e. having more than one antigen binding site). Such multivalent molecules may be specific for one or more antigens. Multivalent molecules of this type may be prepared by dimerization of
25 two antibody fragments through a cysteinyl-containing peptide as, for example disclosed by (Adams *et al.*, 1993; Cumber *et al.*, 1992). Alternatively, dimerization may be facilitated by fusion of the antibody fragments to amphiphilic helices that naturally dimerize (Plunckthun, 1992) or by use of domains (such as leucine zippers *jun* and *fos*)

that preferentially heterodimerize (Kostelny *et al.*, 1992).

The present invention further encompasses chemical analogues of amino acids in the subject antibodies. The use of chemical analogues of amino acids is useful *inter alia* to stabilize the molecules such as if required to be administered to a subject. The analogues of the amino acids contemplated herein include, but are not limited to, modifications of side chains, incorporation of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the proteinaceous molecule or their analogues.

Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with NaBH_4 ; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with NaBH_4 .

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic

- 31 -

anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

5

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

10

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

15 Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acid,
20 contemplated herein is shown in Table 2.

TABLE 2

	Non-conventional amino acid	Code	Non-conventional amino acid	Code
5	α -aminobutyric acid	Abu	L-N-methylalanine	Nmala
	α -amino- α -methylbutyrate	Mgab	L-N-methylarginine	Nmarg
	aminocyclopropane- carboxylate	Cpro	L-N-methylasparagine	Nmasn
			L-N-methylaspartic acid	Nmasp
10	aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
	aminonorbornyl- carboxylate	Norb	L-N-methylglutamine	Nmgln
			L-N-methylglutamic acid	Nmglu
	cyclohexylalanine	Chexa	L-N-methylhistidine	Nmhis
	cyclopentylalanine	Cpen	L-N-methylisoleucine	Nmile
15	D-alanine	Dal	L-N-methylleucine	Nmleu
	D-arginine	Darg	L-N-methyllysine	Nmlys
	D-aspartic acid	Das	L-N-methylmethionine	Nmmet
	D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
	D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
20	D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
	D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
	D-isoleucine	Dile	L-N-methylproline	Nmpro
	D-leucine	Dleu	L-N-methylserine	Nmser
	D-lysine	Dlys	L-N-methylthreonine	Nmthr
25	D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
	D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
	D-phenylalanine	Dphe	L-N-methylvaline	Nmval
	D-proline	Dpro	L-N-methylethylglycine	Nmetg

- 33 -

	D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
	D-threonine	Dthr	L-norleucine	Nle
	D-tryptophan	Dtrp	L-norvaline	Nva
	D-tyrosine	Dtyr	α -methyl-aminoisobutyrate	Maib
5	D-valine	Dval	α -methyl- γ -aminobutyrate	Mgabu
	D- α -methylalanine	Dmala	α -methylcyclohexylalanine	Mchexa
	D- α -methylarginine	Dmarg	α -methylcyclopentylalanine	Mcpen
	D- α -methylasparagine	Dmasn	α -methyl- α -naphthylalanine	Manap
	D- α -methylaspartate	Dmasp	α -methylpenicillamine	Mpen
10	D- α -methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
	D- α -methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
	D- α -methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
	D- α -methylisoleucine	Dmile	N-amino- α -methylbutyrate	Nmaabu
	D- α -methyllleucine	Dmleu	α -naphthylalanine	Anap
15	D- α -methyllysine	Dmlys	N-benzylglycine	Nphe
	D- α -methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
	D- α -methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
	D- α -methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
	D- α -methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
20	D- α -methylserine	Dmser	N-cyclobutylglycine	Ncbut
	D- α -methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
	D- α -methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
	D- α -methyltyrosine	Dmtty	N-cyclodecylglycine	Ncdec
	D- α -methylvaline	Dmval	N-cylcododecylglycine	Ncdod
25	D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
	D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
	D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund

	D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
	D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
	D-N-methylglutamine	Dnmglu	N-(3-guanidinopropyl)glycine	Narg
	D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
5	D-N-methylhistidine	Dnmhis	N-(hydroxyethyl)glycine	Nser
	D-N-methylisoleucine	Dnmile	N-(imidazolylethyl)glycine	Nhis
	D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
	D-N-methyllysine	Dnmlys	N-methyl- γ -aminobutyrate	Nmgabu
	N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmt
10	D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpn
	N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
	N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
	N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
	N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
15	D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
	D-N-methyltyrosine	Dnmtyr	N-methyl- α -naphthylalanine	Nmanap
	D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
	γ -aminobutyric acid	Gabu	N-(<i>p</i> -hydroxyphenyl)glycine	Nhtyr
	L- <i>t</i> -butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
20	L-ethylglycine	Etg	penicillamine	Pen
	L-homophenylalanine	Hphe	L- α -methylalanine	Mala
	L- α -methylarginine	Marg	L- α -methylasparagine	Masn
	L- α -methylaspartate	Masp	L- α -methyl- <i>t</i> -butylglycine	Mtbug
	L- α -methylcysteine	Mcys	L-methylethylglycine	Metg
25	L- α -methylglutamine	Mglu	L- α -methylglutamate	Mglu
	L- α -methylhistidine	Mhis	L- α -methylhomophenylalanine	Mhphe
	L- α -methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet

- 35 -

	L- α -methylleucine	Mleu	L- α -methyllysine	Mlys
	L- α -methylmethionine	Mmet	L- α -methylnorleucine	Mnle
	L- α -methylnorvaline	Mnva	L- α -methylornithine	Morn
	L- α -methylphenylalanine	Mphe	L- α -methylproline	Mpro
5	L- α -methylserine	Mser	L- α -methylthreonine	Mthr
	L- α -methyltryptophan	Mtrp	L- α -methyltyrosine	Mtyr
	L- α -methylvaline	Mval	L-N-methylhomophenylalanine	Nmhpe
	N-(N-(2,2-diphenylethyl)	Nnbhm	N-(N-(3,3-diphenylpropyl)	Nnbhe
	carbamylmethyl)glycine		carbamylmethyl)glycine	
10	1-carboxy-1-(2,2-diphenyl-	Nmbc		
	ethylamino)cyclopropane			

Crosslinkers can be used, for example, to stabilize 3D conformations, using homo-
15 bifunctional crosslinkers such as the bifunctional imido esters having (CH₂)_n spacer
groups with n=1 to n=6, glutaraldehyde, N-hydroxysuccinimide esters and hetero-
bifunctional reagents which usually contain an amino-reactive moiety such as N-
hydroxysuccinimide and another group specific-reactive moiety such as maleimido or
20 dithio moiety (SH) or carbodiimide (COOH). In addition, peptides can be
conformationally constrained by, for example, incorporation of C α and N α -methylamino
acids, introduction of double bonds between C α and C β atoms of amino acids and the
formation of cyclic peptides or analogues by introducing covalent bonds such as forming
an amide bond between the N and C termini, between two side chains or between a side
chain and the N or C terminus.

25

The present invention further contemplates an assay to detect LM04 including the steps
of:-

- 36 -

(3) contacting a monoclonal antibody specific to LM04 or an antigenic determinant thereon with a biological sample suspected of containing a cell containing said LM04; and

5 (4) subjecting the complex formed in step (1) to a signal detection step.

The signal detection step may include ELISA or any other reporter molecule based assays. As part of this detection step, the signal may first need to be amplified.

10 A deimmunized monoclonal antibody of the present invention may also be useful for cancer imaging *in vivo* as well as for targeting cancer cells in order to bring the cancer cells into contact with cell growth retarding or cell killing agents, i.e. cytostatic or cytotoxic agents.

15 With respect to cancer imaging, a reporter molecule is attached to the deimmunized monoclonal antibody and this is then introduced to a host, such as a human. By detecting the reporter molecule, cancer growths can be visualized. One particularly useful form of reporter molecule is a nuclear tag.

20 Accordingly, another aspect of the present invention contemplates a method for detecting neoplastic cells in a human patient, said method comprising introducing into said patient a deimmunized form of a non-human derived monoclonal antibody specific for human LM04 or an antigenic determinant thereon labelled with a reporter molecule, allowing dissemination of the labelled antibody throughout the circulatory system, or to selected
25 parts of the circulatory system and then subjecting said patient to reporter molecule-detection means to identify the location of the antibody.

Immunological based LM04 detection protocols may take a variety of forms. For

example, a plurality of antibodies may be immobilized in an array each with different specificities to particular antigens or cancer cells including LM04. Cells from a biopsy are then brought into contact with the antibody array and a diagnosis may be made as to the type of cancer based on the cells which are immobilized.

5

Other more conventional assays may also be conducted such as by ELISA, Western blot analysis, immunoprecipitation analysis, immunofluorescence analysis, immunochemistry analysis or FACS analysis.

10 The present invention provides, therefore, a method of detecting, in a sample, LM04 or fragment, variant or derivative thereof comprising contacting the sample with an antibody or fragment or derivative thereof and detecting the level of a complex comprising said antibody and LM04 or fragment, variant or derivative thereof compared to normal controls wherein elevated levels of LM04 is indicative of cancer growth.

15

As discussed above, any suitable technique for determining formation of the complex may be used. For example, an antibody according to the invention, having a reporter molecule associated therewith, may be utilized in immunoassays. Such immunoassays include but are not limited to radioimmunoassays (RIAs), enzyme-linked immunosorbent
20 assays (ELISAs) and immunochromatographic techniques (ICTs), Western blotting which are well known to those of skill in the art. For example, reference may be made to "Current Protocols in Immunology", 1994 which discloses a variety of immunoassays which may be used in accordance with the present invention. Immunoassays may include competitive assays. It will be understood that the present invention encompasses
25 qualitative and quantitative immunoassays.

Suitable immunoassay techniques are described, for example, in U.S. Patent Nos. 4,016,043, 4,424,279 and 4,018,653. These include both single-site and two-site assays of

- 38 -

the non-competitive types, as well as the traditional competitive binding assays. These assays also include direct binding of a labelled antigen-binding molecule to a target antigen. The antigen in this case is LM04 or a fragment thereof.

5 Two-site assays are particularly favoured for use in the present invention. A number of variations of these assays exist, all of which are intended to be encompassed by the present invention. Briefly, in a typical forward assay, an unlabelled antigen-binding molecule such as an unlabelled antibody is immobilized on a solid substrate and the sample to be tested brought into contact with the bound molecule. After a suitable period
10 of incubation, for a period of time sufficient to allow formation of an antibody-antigen complex, another antigen-binding molecule, suitably a second antibody specific to the antigen, labelled with a reporter molecule capable of producing a detectable signal is then added and incubated, allowing time sufficient for the formation of another complex of antibody-antigen-labelled antibody. Any unreacted material is washed away and the
15 presence of the antigen is determined by observation of a signal produced by the reporter molecule. The results may be either qualitative, by simple observation of the visible signal, or may be quantitated by comparing with a control sample containing known amounts of antigen. Variations on the forward assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound
20 antibody. These techniques are well known to those skilled in the art, including minor variations as will be readily apparent.

In the typical forward assay, a first antibody having specificity for the antigen or antigenic parts thereof is either covalently or passively bound to a solid surface. The solid
25 surface is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs of microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well known

- 39 -

in the art and generally consist of cross-linking covalently binding or physically adsorbing, the polymer-antibody complex is washed in preparation for the test sample. An aliquot of the sample to be tested is then added to the solid phase complex and incubated for a period of time sufficient and under suitable conditions to allow binding of
5 any antigen present to the antibody. Following the incubation period, the antigen-antibody complex is washed and dried and incubated with a second antibody specific for a portion of the antigen. The second antibody has generally a reporter molecule associated therewith that is used to indicate the binding of the second antibody to the antigen. The amount of labelled antibody that binds, as determined by the associated
10 reporter molecule, is proportional to the amount of antigen bound to the immobilized first antibody.

An alternative method involves immobilizing the antigen in the biological sample and then exposing the immobilized antigen to specific antibody that may or may not be
15 labelled with a reporter molecule. Depending on the amount of target and the strength of the reporter molecule signal, a bound antigen may be detectable by direct labelling with the antibody. Alternatively, a second labelled antibody, specific to the first antibody is exposed to the target-first antibody complex to form a target-first antibody-second antibody tertiary complex. The complex is detected by the signal emitted by the reporter
20 molecule.

From the foregoing, it will be appreciated that the reporter molecule associated with the antigen-binding molecule may include the following:-

- 25 (a) direct attachment of the reporter molecule to the antibody;

- 40 -

(b) indirect attachment of the reporter molecule to the antibody; i.e., attachment of the reporter molecule to another assay reagent which subsequently binds to the antibody; and

5 (c) attachment to a subsequent reaction product of the antibody.

The reporter molecule may be selected from a group including a chromogen, a catalyst, an enzyme, a fluorochrome, a chemiluminescent molecule, a paramagnetic ion, a lanthanide ion such as Europium (Eu^{34}), a radioisotope including other nuclear tags and a
10 direct visual label.

In the case of a direct visual label, use may be made of a colloidal metallic or non-metallic particle, a dye particle, an enzyme or a substrate, an organic polymer, a latex particle, a liposome, or other vesicle containing a signal producing substance and the like.
15

A large number of enzymes suitable for use as reporter molecules is disclosed in U.S. Patent Nos. U.S. 4,366,241, U.S. 4,843,000, and U.S. 4,849,338. Suitable enzymes useful in the present invention include alkaline phosphatase, horseradish peroxidase, luciferase, β -galactosidase, glucose oxidase, lysozyme, malate dehydrogenase and the like. The
20 enzymes may be used alone or in combination with a second enzyme that is in solution.

Suitable fluorochromes include, but are not limited to, fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), R-Phycoerythrin (RPE), and Texas Red. Other exemplary fluorochromes include those discussed by Dower *et al.*, International
25 Publication No. WO 93/06121. Reference also may be made to the fluorochromes described in U.S. Patent Nos. 5,573,909 (Singer *et al.*), 5,326,692 (Brinkley *et al.*). Alternatively, reference may be made to the fluorochromes described in U.S. Patent Nos.

- 41 -

5,227,487, 5,274,113, 5,405,975, 5,433,896, 5,442,045, 5,451,663, 5,453,517, 5,459,276, 5,516,864, 5,648,270 and 5,723,218.

In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognized, however, a wide variety of different conjugation techniques exist which are readily available to the skilled artisan. The substrates to be used with the specific enzymes are generally chosen for the production of, upon hydrolysis by the corresponding enzyme, a detectable colour change. Examples of suitable enzymes include those described *supra*. It is also possible to employ fluorogenic substrates, which yield a fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first antibody-antigen complex, allowed to bind, and then the excess reagent washed away. A solution containing the appropriate substrate is then added to the complex of antibody-antigen-antibody. The substrate will react with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an indication of the amount of antigen which was present in the sample.

Alternately, fluorescent compounds, such as fluorescein, rhodamine and the lanthanide, europium (EU), may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state of excitability in the molecule, followed by emission of the light at a characteristic colour visually detectable with a light microscope. The fluorescent-labelled antibody is allowed to bind to the first antibody-antigen complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to light of an appropriate wavelength. The fluorescence observed indicates the presence of the antigen of interest. Immunofluorometric assays (IFMA) are well established in the art and are particularly

- 42 -

useful for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules may also be employed.

5 In another embodiment, the method for detection comprises detecting the level of expression in a cell of a polynucleotide encoding a LM04. Expression of said polynucleotide may be determined using any suitable technique. For example, a labelled polynucleotide encoding a LM04 may be utilized as a probe in a Northern blot of an RNA extract obtained from the cell. Preferably, a nucleic acid extract from the animal is utilized in concert with oligonucleotide primers corresponding to sense and antisense
10 sequences of a polynucleotide encoding the kinase, or flanking sequences thereof, in a nucleic acid amplification reaction such as RT PCR. A variety of automated solid-phase detection techniques are also appropriate. For example, a very large scale immobilized primer arrays (VLSIPS™) are used for the detection of nucleic acids as, for example, described by Fodor *et al.*, 1991 and Kazal *et al.*, 1996. The above genetic techniques are
15 well known to persons skilled in the art.

For example, to detect LM04 encoding RNA transcripts, RNA is isolated from a cellular sample suspected of containing LM04 RNA, e.g. total RNA isolated from human breast cancer tissue. RNA can be isolated by methods known in the art, e.g. using TRIZOL™
20 reagent (GIBCO-BRL/Life Technologies, Gaithersburg, Md.). Oligo-dT, or random-sequence oligonucleotides, as well as sequence-specific oligonucleotides can be employed as a primer in a reverse transcriptase reaction to prepare first-strand cDNAs from the isolated RNA. Resultant first-strand cDNAs are then amplified with sequence-specific oligonucleotides in PCR reactions to yield an amplified product.

25

“Polymerase chain reaction” or “PCR” refers to a procedure or technique in which amounts of a preselected fragment of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Patent No. 4,683,195. Generally, sequence information from the ends of

the region of interest or beyond is employed to design oligonucleotide primers. These primers will be identical or similar in sequence to opposite strands of the template to be amplified. PCR can be used to amplify specific RNA sequences and cDNA transcribed from total cellular RNA. See generally Mullis *et al.*, 1987; Erlich, 1989. Thus, amplification of specific nucleic acid sequences by PCR relies upon oligonucleotides or “primers” having conserved nucleotide sequences wherein the conserved sequences are deduced from alignments of related gene or protein sequences, e.g. a sequence comparison of mammalian LM04 genes. For example, one primer is prepared which is predicted to anneal to the antisense strand and another primer prepared which is predicted to anneal to the sense strand of a cDNA molecule which encodes LM04.

To detect the amplified product, the reaction mixture is typically subjected to agarose gel electrophoresis or other convenient separation technique and the relative presence of the LM04 specific amplified DNA detected. For example, LM04 amplified DNA may be detected using Southern hybridization with a specific oligonucleotide probe or comparing is electrophoretic mobility with DNA standards of known molecular weight. Isolation, purification and characterization of the amplified LM04 DNA may be accomplished by excising or eluting the fragment from the gel (for example, see references Lawn *et al.*, 1981; Goeddel *et al.*, 1980), cloning the amplified product into a cloning site of a suitable vector, such as the pCRII vector (Invitrogen), sequencing the cloned insert and comparing the DNA sequence to the known sequence of LM04. The relative amounts of LM04 mRNA and cDNA can then be determined.

The present invention may be used to detect any neoplasm which comprises cells which express elevated levels of LM04.

The common medical meaning of the term “neoplasia” refers to “new cell growth” that results as a loss of responsiveness to normal growth controls, e.g. to neoplastic cell

growth. A "hyperplasia" refers to cells undergoing an abnormally high rate of growth. However, as used herein, the terms "neoplasia" and "hyperplasia" can be used interchangeably, referring generally to cells experiencing abnormal cell growth rates. Neoplasia and hyperplasia include "tumors" which may be either benign, pre-malignant or malignant.

As used herein, the terms "hyperproliferative" and "neoplastic" are used interchangeably and refer to those cells in an abnormal state or condition characterized by rapid proliferation or neoplasm. The terms are meant to include all types of cancerous growths or oncogenic processes, metastatic tissues or malignantly transformed cells, tissues or organs irrespective of histopathologic type or state of invasiveness. "Pathologic hyperproliferative" cells occur in disease states characterized by malignant tumor growth.

The term "carcinoma" is recognized by those skilled in the art and refers to malignancies of epithelial or endocrine tissues including respiratory system carcinomas, gastrointestinal system carcinomas, genitourinary system carcinomas, testicular carcinomas, breast carcinomas, prostatic carcinomas, endocrine system carcinomas and melanomas. Exemplary carcinomas include those forming from tissue of the breast. The term also includes carcinosarcomas, e.g. which include malignant tumors composed of carcinomatous and sarcomatous tissues. An "adenocarcinoma" refers to a carcinoma derived from glandular tissue or in which the tumor cells form recognizable glandular structures.

As detailed hereinbefore the term "neoplasm" as used herein encompasses all the terms discussed in the preceding three paragraphs.

The method of the present invention is useful as a one off test or as an on-going monitor of those individuals thought to be at risk of neoplasm development or as a monitor of the

- 45 -

effectiveness of therapeutic or prophylactic treatment regimes directed to inhibiting or otherwise slowing neoplasm development, in particular, breast cancer development. In these situation, mapping the modulation of LM04 levels in any one or more classes of biological samples is a valuable indicator of the status of an individual or the effectiveness of a therapeutic or prophylactic regime which is currently in use. Accordingly, the method of the present invention should be understood to extend to monitoring for increases or decreases in marker levels in an individual relative to their normal level (as hereinbefore defined) or relative to one or more earlier marker levels determined from a biological sample of said individual.

The present invention further contemplates the use of a monoclonal antibody to LM04 in the manufacture of a quantitative or semi-quantitative diagnostic kit to determine relative levels of LM04 in suspected neoplastic cells from a patient. The kit may come with instructions for use and may be automated or semi-automated or in a form which is compatible with automated machine or software.

The generation of antibodies to LM04 may, in accordance with the present invention, be directed to the active or inactive forms of the molecule. Antibodies directed to an active LM04 are particularly useful in detecting an increase or decrease in LM04 activity.

The identification of LM04 as a cancer-specific molecule, in particular a breast cancer specific molecule, permits the generation of targeting agents to destroy or at least retard the growth of the cancer cells. In particular, the cancer targeting agents comprising LM04 specific antibodies are fused, bound or otherwise associated with a cell growth inhibiting or killing agent. Such agents include but are not limited to cytocidal or cytostatic agents which act at the protein or corresponding mRNA or DNA levels. For example, the cell growth or killing agent maybe a nuclear tag or may be an agent which promotes induction of antagonists of LM04 RNAi or RNA oligonucleotides.

- 46 -

Accordingly, one aspect of the present invention provides a method of modulating LM04 regulated cellular proliferation, said method comprising contacting said cell with an effective amount of an agent for a time and under conditions sufficient to modulate *LM04* expression or LM04 functional activity wherein inhibiting or otherwise antagonising said expression or activity down-regulates said cellular proliferation.

Reference to "LM04 regulated cellular proliferation" should be understood as a reference to proliferative activity which is either directly or indirectly induced or otherwise regulated by LM04.

Without limiting the present invention to any one theory or mode of action, up-regulation of LM04 expression has been identified as a marker of uncontrolled breast cell proliferation, in particular epithelial breast cell proliferation. However, it should be understood that the diagnostic, therapeutic and prophylactic methodology disclosed herein should not be limited to uncontrolled breast cell proliferation but extended to any cell type, the proliferation of which is directly or indirectly regulated by LM04.

The present invention more particularly provides a method of modulating LM04-regulated mammary cell proliferation, said method comprising contacting said cell with an effective amount of an agent for a time and under conditions sufficient to modulate *LM04* expression or LM04 functional activity wherein inhibiting or otherwise antagonising said expression or activity down-regulates said cellular proliferation.

It should be understood that antagonism LM04 expression or activity may be partial or complete. Partial modulation occurs where only some LM04 expression or activity occurring in a given cell is down-regulated. LM04 expression or functional activity is down-regulated.

Down-regulation of *LM04* expression or LM04 functional activity may be achieved by any one of a number of techniques including, but not limited to:

- 5 (i) introducing into a cell a proteinaceous or non-proteinaceous molecule which modulates the transcriptional and/or translational regulation of the LM04 gene or a gene, the expression product of which regulates LM04 expression.
- 10 (ii) introducing into a cell a proteinaceous or non-proteinaceous molecule which antagonises the interaction between LM04 and one or more of its ligands (for example, bdb1, Nb1, CL1M, CtIP or BRCA1).

Up-regulation of LM04 may also be desired in certain circumstances and may be achieved by introducing into a cell, LM04, *LM04* or derivative, chemical equivalent, homologue or mimetic thereof or a proteinaceous or non-proteinaceous molecule which
15 agonises the interaction between LM04 and its ligands or otherwise upregulates its activity or *LM04* expression.

Reference to "agent" should be understood as a reference to any proteinaceous or non-proteinaceous molecule which achieves the above objectives and includes, for example,
20 the molecules detailed in the points above. The subject agent may be linked, bound or otherwise associated with any proteinaceous or non-proteinaceous molecule. For example, it may be associated with a molecule which permits its targeting to a localised region, for example the breast.

25 Said proteinaceous molecule may be derived from natural, recombinant or synthetic sources including fusion proteins or following, for example, natural product screening. Said non-proteinaceous molecule may be derived from natural sources, such as for example natural product screening or may be chemically synthesised. The present

invention contemplates chemical analogues of said LM04 capable of acting as antagonists of LM04 interactions. Antagonists may be any compound capable of blocking, inhibiting or otherwise preventing said LM04 from interacting. Antagonists include monoclonal antibodies specific for said LM04, or parts of said LM04, and antisense nucleic acids which prevent transcription or translation of genes or mRNA in the subject cells. Modulation of expression may also be achieved utilising antigens, RNA, ribosomes, DNAzymes, RNA aptamers, antibodies or molecules suitable for use in co-suppression or to induce RNAi-mediated down-regulation of LM04 mRNA transcript.

10 Synthetic sources of said agent include for example chemically synthesised molecules. In other examples, phage display libraries can be screened for peptides while chemical libraries can be screened for existing small molecules. Rational drug design/structure based design can be achieved by performing crystallisation, further analysing the LM04 binding sites and fitting molecules into that site by design.

15 For example chemical or functional equivalents of LM04 can be designed and/or identified utilising well known methods such as combinatorial chemistry or high throughput screening of recombinant libraries or following natural product screening.

20 In another example, libraries containing small organic molecules may be screened, wherein organic molecules having a large number of specific parent group substitutions are used. A general synthetic scheme may follow published methods (e.g. Bunin *et al.*, 1994; DeWitt *et al.*, 1993). Briefly, at each successive synthetic step, one of a plurality of different selected substituents is added to each of a selected subset of tubes in an array, with the selection of tube subsets being such as to generate all possible permutation of the different substituents employed in producing the library. One suitable permutation strategy is outlined in US. Patent No. 5,763,263.

There is currently widespread interest in using combinational libraries of random organic molecules to search for biologically active compounds (see for example U.S. Patent No. 5,763,263). Ligands discovered by screening libraries of this type may be useful in mimicking or blocking natural ligands or interfering with the naturally occurring ligands of a biological target. In the present context, for example, they may be used as a starting point for developing analogues which exhibit properties such as more potent pharmacological effects. LM04 or a functional part thereof may according to the present invention be used in combination libraries formed by various solid-phase or solution-phase synthetic methods (see for example U.S. Patent No. 5,763,263 and references cited therein). By use of techniques, such as that disclosed in U.S. Patent No. 5,753,187, millions of new chemical and/or biological compounds may be routinely screened in less than a few weeks. Of the large number of compounds identified, only those exhibiting appropriate biological activity are further analysed.

With respect to high throughput library screening methods, oligomeric or small-molecule library compounds capable of interacting specifically with a selected biological agent, such as a biomolecule, a macromolecule complex, or cell, are screened utilising a combinational library device which is easily chosen by the person of skill in the art from the range of well-known methods, such as those described above. In such a method, each member of the library is screened for its ability to interact specifically with the selected agent. In practising the method, a biological agent is drawn into compound-containing tubes and allowed to interact with the individual library compound in each tube. The interaction is designed to produce a detectable signal that can be used to monitor the presence of the desired interaction. Preferably, the biological agent is present in an aqueous solution and further conditions are adapted depending on the desired interaction. Detection may be performed for example by any well-known functional or non-functional based method for the detection of substances.

- 50 -

In addition to screening for molecules which mimic the activity of LM04, it may also be desirable to identify and utilise molecules which function agonistically or antagonistically in order to up or down-regulate the functional activity of the LM04. The use of such molecules is described in more detail below. To the extent that the subject molecule is proteinaceous, it may be derived, for example, from natural or recombinant sources including fusion proteins or following, for example, the screening methods described above. The non-proteinaceous molecule may be, for example, a chemical or synthetic molecule which has also been identified or generated in accordance with the methodology identified above. Accordingly, the present invention contemplates the use of chemical analogues capable of acting as agonists or antagonists. Chemical agonists may not necessarily be derived from LM04 but may share certain conformational similarities. Alternatively, chemical agonists may be specifically designed to mimic certain physiochemical properties of LM04.

Screening for the modulatory agents hereinbefore defined can be achieved by any one of several suitable methods including, but in no way limited to, contacting a cell comprising LM04 with an agent and screening for the modulation of LM04-related functional activity or modulation of the activity or expression of a downstream LM04 cellular target. Detecting such modulation can be achieved utilising techniques such as Western blotting, electrophoretic mobility shift assays and/or the readout of reporters of sphingosine kinase or TRAF activity such as luciferases, CAT and the like.

It should be understood that the LM04 protein may be naturally occurring in the cell which is the subject of testing or the gene encoding it may have been transfected into a host cell for the purpose of testing. Further, the naturally occurring or transfected gene may be constitutively expressed - thereby providing a model useful for, *inter alia*, screening for agents which down-regulate LM04 expression or activity or the gene may require activation - thereby providing a model useful for, *inter alia*, screening for agents

- 51 -

which modulate LM04 expression or activity under certain stimulatory conditions. Further, to the extent that a LM04 nucleic acid molecule is transfected into a cell, that molecule may comprise the entire LM04 gene or it may merely comprise a portion of the gene such as the LM04 binding portion.

5

In another example, the subject of detection could be a downstream LM04 regulatory target, rather than LM04 itself, such as CtIP or BRCA1. Yet another example includes LM04 binding sites ligated to a minimal reporter. This is an example of a system where modulation of the molecules which LM04 regulates the activity of, are monitored. Where
10 the cell which is the subject of the screening system is a neoplastic cell, for example, modulation of LM04 expression or activity could be detected by screening for the modulation of cellular proliferation.

Accordingly, another aspect of the present invention provides a method for detecting an
15 agent capable of modulating *LM04* expression or LM04 functional activity said method comprising contacting a cell or extract thereof containing said *LM04* or LM04 with a putative agent and detecting an altered expression phenotype associated with said interaction.

20 Reference to detecting an "altered expression phenotype associated with said interaction" should be understood as the detection of cellular changes associated with modulation of *LM04* expression or LM04 activity. These may be detectable, for example, as intracellular changes or changes observable extracellularly. For example, this includes, but is not limited to, detecting changes in downstream product levels or activities.

25

In a related aspect, the present invention should be understood to extend to the agents identified utilising any of the methods hereinbefore defined. In this regard, reference to

- 52 -

an agent should be understood as a reference to any proteinaceous or non-proteinaceous molecule which modulates at least one LM04-related functional activity.

Said proteinaceous or non-proteinaceous molecule may act either directly or indirectly to modulate *LM04* expression or LM04 activity. Said molecule acts directly if it associates with LM04 molecules. Said molecule acts indirectly if it associates with a molecule other than LM04, which other molecule either directly or indirectly modulates the expression or activity of LM04. Accordingly, the method of the present invention encompasses regulation of LM04 expression or activity via the induction of a cascade of regulatory steps.

Although the preferred method is to down-regulate *LM04* expression or LM04 functional activity, it should be understood that there may be situations in which it is desirable to up-regulate proliferation, in a controlled manner for example, by up-regulating *LM04* expression or LM04 functional activity. Accordingly, the method of the present invention should be understood to extend to such applications.

It should be understood that the cell which is treated according to the method of the present invention may be located *ex vivo* or *in vivo*. By "*ex vivo*" is meant that the cell has been removed from the body of a subject wherein the modulation of its proliferation will be initiated *in vitro*. For example, the cell may be a non-neoplastic cell which is to undergo long term culture and is therefore stimulated to undergo ongoing LM04 induced proliferation, which proliferation is up-regulated by agonists of LM04 activity. In accordance with the preferred aspects of the present invention, the cell may be a neoplastic cell, such as a malignant cell, located *in vivo* (such as in the breast) and the down-regulation of its growth will be achieved by applying the method of the present invention *in vivo* to down-regulate the level of LM04.

A further aspect of the present invention relates to the use of the invention in relation to the treatment and/or prophylaxis of disease conditions. Without limiting the present invention to any one theory or mode of action, LM04 has been shown to regulate mammary cell proliferation. Accordingly, the method of the present invention provides a valuable tool for modulating aberrant or otherwise unwanted LM04-regulated proliferative activity.

Accordingly, another aspect of the present invention is directed to the method for the treatment and/or prophylaxis of a conditions characterised by aberrant, unwanted or otherwise inappropriate LM04-regulated proliferative activity in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate *LM04* expression or LM04 functional activity wherein inhibiting or otherwise antagonising said expression or activity down-regulates said cellular proliferation.

Reference to "aberrant, unwanted or otherwise inappropriate" cellular proliferation should be understood as a reference to overactive cellular proliferation, to physiologically normal cellular proliferation which is inappropriate in that it is unwanted or to insufficient cellular proliferation. For example, LM04 expression in mammary cells is thought to lead to deregulation of differentiation and subsequently to uncontrolled breast cell proliferation.

Accordingly, in one preferred embodiment there is provided a method for the treatment and/or prophylaxis of a neoplastic condition said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to down-regulate *LM04* expression or LM04 functional activity wherein inhibiting or otherwise antagonising said expression or activity down-regulates cellular proliferation.

- 54 -

Preferably, said neoplastic condition is breast cancer.

The term "mammal" as used herein includes humans, primates, livestock animals (eg. sheep, pigs, cattle, horses, donkeys), laboratory test animals (eg. mice, rabbits, rats, guinea pigs), companion animals (eg. dogs, cats) and captive wild animals (eg. foxes, kangaroos, deer). Preferably, the mammal is human or a laboratory test animal. Even more preferably, the mammal is a human.

An "effective amount" means an amount necessary at least partly to attain the desired response, or to delay the onset or inhibit progression or halt altogether, the onset or progression of a particular condition being treated. The amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated, the degree of protection desired, the formulation of the composition, the assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

Reference herein to "treatment" and "prophylaxis" is to be considered in its broadest context. The term "treatment" does not necessarily imply that a subject is treated until total recovery. Similarly, "prophylaxis" does not necessarily mean that the subject will not eventually contract a disease condition. Accordingly, treatment and prophylaxis include amelioration of the symptoms of a particular condition or preventing or otherwise reducing the risk of developing a particular condition. The term "prophylaxis" may be considered as reducing the severity or onset of a particular condition. "Treatment" may also reduce the severity of an existing condition.

- 55 -

The present invention further contemplates a combination of therapies, such as the administration of the agent together with subjection of the mammal to circulating cytotoxic agents or to radiotherapy in the treatment of cancer.

5 Administration of the modulatory agent, in the form of a pharmaceutical composition, may be performed by any convenient means. The modulatory agent of the pharmaceutical composition is contemplated to exhibit therapeutic activity when administered in an amount which depends on the particular case. The variation depends, for example, on the human or animal and the modulatory agent chosen. A broad range of
10 doses may be applicable. Considering a patient, for example, from about 0.1 mg to about 1 mg of modulatory agent may be administered per kilogram of body weight per day. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or other suitable time intervals or the dose may be proportionally reduced as indicated by the
15 exigencies of the situation.

The modulatory agent may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intraperitoneal, intramuscular, subcutaneous, intradermal or suppository routes or implanting (e.g. using slow release molecules). The
20 modulatory agent may be administered in the form of pharmaceutically acceptable nontoxic salts, such as acid addition salts or metal complexes, e.g. with zinc, iron or the like (which are considered as salts for purposes of this application). Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, maleate, acetate, citrate, benzoate, succinate, malate, ascorbate, tartrate and the like. If the active
25 ingredient is to be administered in tablet form, the tablet may contain a binder such as tragacanth, corn starch or gelatin; a disintegrating agent, such as alginic acid; and a lubricant, such as magnesium stearate.

- 56 -

Routes of administration include, but are not limited to, respiratorally, intratracheally, nasopharyngeally, intravenously, intraperitoneally, subcutaneously, intracranially, intradermally, intramuscularly, intraocularly, intrathecally, intracerebrally, intranasally, infusion, orally, rectally, *via* IV drip patch and implant.

5

In accordance with these methods, the agent defined in accordance with the present invention may be coadministered with one or more other compounds or molecules. By "coadministered" is meant simultaneous administration in the same formulation or in two different formulations via the same or different routes or sequential administration by the same or different routes. For example, the subject agent may be administered together with an agonistic agent in order to enhance its effects. By "sequential" administration is meant a time difference of from seconds, minutes, hours or days between the administration of the two types of molecules. These molecules may be administered in any order.

15

Another aspect of the present invention contemplates the use of an agent, as hereinbefore defined, in the manufacture of medicament for the treatment of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate LM04-regulated cellular proliferation, wherein said agent modulates *LM04* expression or LM04 activity and wherein inhibiting or otherwise antagonising said expression or activity down-regulates said cellular proliferation.

20

In yet another further aspect, the present invention contemplates a pharmaceutical composition comprising the modulatory agent as hereinbefore defined together with one or more pharmaceutically acceptable carriers and/or diluents. Said agents are referred to as the active ingredients.

25

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion or may be in the form of a cream or other form suitable for topical application. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilisation. Generally, dispersions are prepared by incorporating the various sterilised active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit.

10 The amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 μ g and 2000 mg of active compound.

15 The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent,

20 methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition,

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- 59 -

the active compound(s) may be incorporated into sustained-release preparations and formulations.

5 The pharmaceutical composition may also comprise genetic molecules such as a vector capable of transfecting target cells where the vector carries a nucleic acid molecule encoding a modulatory agent. The vector may, for example, be a viral vector.

Yet another aspect of the present invention relates to the agent as hereinbefore defined, when used in the method of the present invention.

10

The present invention is further defined by the following non-limiting examples:

- 60 -

EXAMPLE 1
THE LIM DOMAIN PROTEIN LM04 INHIBITS DIFFERENTIATION OF
MAMMARY EPITHELIAL CELLS *IN VITRO* AND IS OVEREXPRESSED IN
BREAST CANCER

Cell Lines

Most of the cell lines have been cited in previous studies (20). SCp2 mammary epithelial cells (21) were kindly provided by Dr M Bissell.

***In situ* Hybridisation**

Mouse LM04 was isolated from mouse T cells by affinity chromatography using rabbit anti-Ldb1 antisera (19). The purification and cloning of LM04 will be described elsewhere (K. H.). Full-length human (from ATCC, Acc. No. T09407) or mouse LM04 cDNAs were cloned into Bluescript SKII (Stratagene). Antisense and sense riboprobes were generated using T3 or T7 RNA polymerase (Promega) with digoxigenin-UTP (Roche). Standard *in situ* hybridisations were performed as described (22). Breast cancer tissue arrays were either purchased from Clinomics or prepared from breast cancer samples at the Peter MacCallum Cancer Institute (PMCI), Melbourne, Australia. All specimens evaluated were anonymous, archival tissue specimens. The Peter MacCallum Institutional Review Board approved the use of PMCI specimens for microarray analysis.

SCp2 Cell Differentiation Assay

SCp2 mammary epithelial cells (21) were passaged in DMEM-F12 media containing DMEM-HAM, 10% FCS, and insulin 5 µg/ml (Sigma). Full-length mouse cDNAs corresponding to LM04 or Ldb1 were cloned into both the pEF1α-puro and pEF1α-Flag-

- 61 -

puro mammalian expression vectors (23). Protein expression was confirmed by transient transfection of 293T cells. Linearized expression vectors (10 µg) were introduced into SCp2 cells using Superfect (Qiagen) and selected in puromycin for 8 – 10 days. Pools of stable transfectants expressing the appropriate gene or vector alone (control) were then used in the differentiation assay, essentially described (21). Transfectants were induced to differentiate by the addition of DMEM-F12 containing insulin (5 µg/ml), hydrocortisone (1 µg/ml) and prolactin (5 µg/ml), which was generously provided by Dr A. Parlow (National Hormone and Pituitary Program). After 96 hours, cells were harvested directly for RNA extraction.

RNA Analysis and RT-PCR

Northern analysis of poly(A)⁺ RNA was performed as described (24). Total RNA was isolated from SCp2 cells on ECM using RNazol (Tel-Test); cDNA synthesis and PCR were performed as described (25), using primers for β-casein and HPRT (25). Sequences for the β-casein primers were: forward 5'- ATGAAGGTCTTCATCCTCGCCTGCC-3', reverse 5'- GCTGGACCAGAGACTGAGGAAGGTGC-3'. Samples were fractioned on agarose gels, ethidium bromide stained, blotted, then hybridised with specific internal oligonucleotides for β-casein (5'-TTCTCTCTGGATCCAGGAGTG-3') and HPRT (5'-GCCCTTGACTATAATGAGTACTTCAGGAATTTGAATC-3').

Immunoprecipitation and Western Analysis

Whole cell lysates were generated from stably transfected SCp2 pools by lysing cells in KALB lysis buffer (26) containing protease inhibitors. Proteins were immunoprecipitated with anti-Flag M2 (Sigma) and protein G Sepharose (Pharmacia), and separated by SDS-PAGE (Novex). After transfer, filters were blocked and incubated with rabbit antisera to either full-length mouse LM04 or a C-terminal Ldb1 polypeptide

(19). Antibody binding was visualised with peroxidase-conjugated anti-mouse antibody by ECL (Amersham).

Immunohistochemistry

For immunostaining, tissue arrays (Clinomics) were incubated with supernatant containing rat anti-LM04 monoclonal antibody, followed by incubation with biotinylated anti-rat IgG and HRP-Streptavidin (Dako), before detection using DAB (Dako), as described (27). Generation of the LM04 monoclonal antibody will be described elsewhere. Other primary monoclonal antibodies used were: ER α (1D5), PgR(636) and ErbB2 (all from Dako).

EXAMPLE 2

LM04 IS DEVELOPMENTALLY REGULATED IN THE MOUSE MAMMARY GLAND

In situ hybridisation analysis revealed that LM04 was prominently expressed in the lobuloalveolar units of the mammary gland during pregnancy (Fig. 1A). At this stage, there is a high rate of proliferation accompanying the formation and expansion of the lobuloalveoli. Lower levels of LM04 mRNA were present in the ductal epithelium of the virgin mammary gland, evident as a single layer, and in the lactating and involuting mammary glands. Staining of the surrounding stroma was also apparent in the virgin mammary gland. The high level of LM04 in the pregnant mammary gland (Fig. 1A) was confirmed by Northern analysis, which revealed that levels of the two major transcripts (approximately 1.8 and 2.3 kb) peaked at mid-pregnancy (day 12) and remained high until late pregnancy (Fig. 1B). Interestingly, although LM04 expression decreased during lactation, it appeared to be upregulated during involution.

EXAMPLE 3

FORCED EXPRESSION OF THE LM04 AND LDB1 GENES INHIBITS β - CASEIN SYNTHESIS IN MAMMARY EPITHELIAL CELLS

5 LM04 binds with high affinity to Ldb1, a nuclear protein that serves as an adaptor for several LIM domain-containing proteins. To examine the role of LM04 and its partner protein Ldb1 in mammary differentiation, we introduced the genes into SCp2 mammary epithelial cells, which express moderate levels of LM04 and Ldb1 RNA (Fig. 3). The SCp2 cell line was originally isolated from the mammary gland of a mid-gestation mouse
10 and mimics the essential features of mammary differentiation in the presence of extracellular matrix (ECM) and a lactogenic stimulus (21). Differentiation of SCp2 cells is accompanied by the production of milk proteins, such as β -casein, which we have used here as a molecular marker. Stable transfectants of SCp2 cells harboring LM04 or Ldb1 cDNAs (either epitope-tagged or untagged) together with a puromycin resistance marker,
15 were generated and pools of cells assayed for their ability to undergo differentiation. For the latter assay, transfectants were plated on ECM in the presence or absence of a lactogenic stimulus (prolactin, insulin and hydrocortisone).

Overexpression of LM04 or Ldb1 markedly inhibited β -casein synthesis upon treatment
20 with a lactogenic stimulus, in contrast to transfectants expressing empty vector, which were indistinguishable from parental cells (Fig. 2A). Expression of the Flag-tagged LM04 and Ldb1 proteins was readily detectable in several SCp2 transfectants, examples of which are shown in Fig. 2B, while expression of the untagged transgenes was verified by Northern analysis (data not shown). The inhibition of β -casein synthesis indicates that
25 LM04 and its partner protein Ldb1 play a role in maintaining proliferation rather than differentiation of mammary epithelial cells.

EXAMPLE 4

OVEREXPRESSION OF LM04 IN BREAST CANCER CELL LINES AND PRIMARY INVASIVE CANCERS

5 LM04 RNA levels vary markedly between different human breast epithelial cancer cell lines (Fig. 3). High levels of LM04 mRNA were detected in five out of ten breast cancer cell lines. Interestingly, transcript levels were low in the immortalised cell line 184 but were substantially higher in a transformed variant of this line, 184B5 (20). Ldb1 was expressed as two major transcripts (2.3 and 3.5 kb) amongst the panel of breast cancer
10 cell lines and showed less variation in the extent of expression.

To assess whether LM04 was upregulated in primary breast cancers, we performed *in situ* hybridisation on tissue arrays comprising 177 invasive breast cancers. Strong to moderate staining was evident in 99 of 177 (56%) cancers, most of which corresponded
15 to infiltrating ductal carcinoma but also included lobular and mixed ductal and lobular carcinomas. Fig. 4 shows prominent expression of LM04 in two infiltrating ductal (Fig. 4A, C) and one lobular carcinoma (Fig. 4B), while low levels were detectable in 7 out of 20 (35%) normal breast tissue samples (Fig. 4D). LM04 RNA levels appeared to be elevated in ductal carcinoma *in situ* (DCIS) (Fig. 4E), in which 7 of 18 (38%) pre-
20 invasive tumors showed strong to moderate staining. A subset of 60 tumours were analysed by immunohistochemistry using a rat anti-LM04 monoclonal antibody, revealing overexpression in at least 50% of tumors (Fig. 4f-H). Two examples of *in situ* RNA and immunohistochemical staining for the same tumors (Fig. 4B & F and Fig. 4C & G) are shown. The sense riboprobe and controls for immunohistochemistry gave
25 negligible staining. Subcellular localisation of LM04 was variable; in some tumors, nuclear staining was detected (Fig. 4F), whereas in other tumors, cytoplasmic plus some nuclear staining was visualised (Fig. 4G, H). These findings are consistent with localisation studies of LM04 in transfected cells (12, 13). The same tumor set of 60

- 65 -

samples were evaluated for expression of the Estrogen (ER α), Progesterone and ErbB2 receptors by immunohistochemistry, in which 32%, 40% and 30% were positive for the respective receptors (data not shown). Interestingly, there was an apparent inverse correlation between nuclear LM04 expression and nuclear ER α expression (data not shown). Studies with larger sample numbers will be required to confirm this finding.

EXAMPLE 5

THE LIM DOMAIN PROTEIN LM04 INTERACTS WITH THE COREPRESSOR CtIP AND THE TUMOR SUPPRESSOR BRCA1 AND INHIBITS BRCA1 ACTIVITY

Plasmids

The pGBT9-LMO4 bait plasmid was generated by PCR amplification of mouse LMO4 in pSP72 using the following primers: forward 5'-CGCGGATCCCCGGCTCCCTCTCCTGGAAGCGCTGC-3' and reverse 5'-CGC GGA TCC TCA GCA GAC CTT CTG GTC TGC CAG-3'; the resultant product was inserted into the BamHI site of pGBT9 (Clontech). The first LIM domain (residues 1-82) of LMO4 was PCR amplified using the forward primer 5'-CGCCGATCCTGAATCCGGGCAGCAGCTCGC-3' and the reverse primer 5'-CGCGGATCCTCACCCAAATAACCTAATGTAGTCATT-3', then cloned into the BamHI site of the Flag-pEF1 α -puro vector (19). Expression vectors encoding Lhx1 or Lhx3 (pSV-sport-Flag) and LMK1 were kindly provided by A. Agulnick and O. Bernard, respectively. Full-length cDNAs corresponding to the coding region of mouse or human LMO4 were cloned into the Flag-pEF1 α -puro expression vector, and a 0.7 kb mouse LMO4 cDNA fragment was cloned into pEF1 α -puro vector. The expression plasmids encoding residues 45-897 of human CtIP (pCMV-HA-ns311), human BRCA1 (pcDNA3-HA-BRCA1) and mouse LMO2 (pEF1 α -Flag-LMO2) have previously been described

(Scully *et al.*, 1997; Visvader *et al.*, 1997; Yu *et al.*, 1998). HA-tagged CtIP deletion mutants were generated either by PCR amplification or subcloning from plasmids (Yu *et al.*, 1998), into the expression vectors HA-pcDNA3.1 or HA-EF1 α -puro. The SZ fragment of BRCA1 (residues 1528 - 1863) was recloned from pCMV-Gal4b-BR-SZ (Yu *et al.*, 1998) into HA-pCDNA3.1.

The yeast expression construct encoding the activation domain (AD) of BRCA1 fused to the Gal4 DNA-binding domain (DBD) was generated by PCR amplification of the region spanning amino acids 1293 - 1863 of BRCA1 and subsequent cloning into pGBT9 (Clontech). The mammalian Gal4DBD-AD fusion construct was generated by cloning a cDNA fragment encoding the BRCA1-AD region into pCMV-Gal4b (58). Full-length cDNA encoding mouse LMO4 was inserted into the yeast expression plasmid pYX212 (Ingenious). The reporter plasmid, pG5CAT, was from Clontech. Details of plasmid constructions are available on request.

Yeast two-hybrid screen

The pGBT9-LMO4 bait plasmid (residues 15 - 165) was used to screen 8.4×10^6 transformants from a primary breast adenocarcinoma cDNA library (Byrne *et al.*, 1998), following standard protocols (Clontech Matchmaker Two-Hybrid System).

Antibody production

Full-length mouse LMO4 cDNA was amplified by PCR and subcloned into pGEX-2T (Amersham Pharmacia Biotech.). The GST-LMO4 fusion protein was expressed in the bacterial strain UT5600, purified according to standard protocols (Smith *et al.*, 1988) and used to inject rabbits to produce polyclonal antisera. The generation of rat LMO4-specific monoclonal antibodies will be described elsewhere.

Immunoprecipitation and western blot analysis

Human embryonal kidney 293T cells (10 cm plates) were transiently transfected with 4
5 μ g of each expression construct and/or empty vector using the calcium phosphate
precipitation method. Cell extracts (0.5 ml) were prepared in whole cell lysis buffer (150
mM NaCl, 5 mM EDTA, 50 mM Tris pH7.5, 1% Nonidet P-40, 1 mM DTT) containing
protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, 1 μ g/ml
pepstatin, 1 μ g/ml aprotinin) and normalised for protein concentration, as determined by
10 Bradford analysis (BioRad). Proteins were immunoprecipitated with either anti-Flag M2
(Sigma) or anti-myc 9E10 and protein G sepharose (Amersham Pharmacia Biotech.), and
separated by SDS-PAGE (Novex). After transfer to PVDF membranes (Millipore), filters
were blocked and incubated with rabbit antisera against CtIP or Ldb1 (Visvader *et al.*,
1997) or mouse anti-BRCA1 monoclonal antibody MS110 (Oncogene Research
15 Products). Filters were then incubated with HRP-coupled secondary antibodies, and
developed by ECL (Amersham Pharmacia Biotech.).

For detection of endogenous proteins, nuclear extracts from human breast epithelial
HBL100 cells were prepared (Watters *et al.*, 1997). Proteins were immunoprecipitated
20 with either anti-LMO4 rabbit antisera or a rat anti-LMO4 monoclonal antibody and
protein G sepharose, and fractionated by SDS-PAGE. After transfer, filters were blocked
and incubated with mouse monoclonal antibodies to either CtIP (14.1) (Yu *et al.*, 2000)
or BRCA1 (MS110, Oncogene Research Products), followed by incubation with HRP-
coupled secondary antibodies, and developed by ECL (Amersham Pharmacia Biotech.).

***In vitro* binding assay**

In vitro synthesis of ³⁵S-labelled BRCA1 (C-terminal residues 1528 to 1863) was performed by *in vitro* transcription/translation of HA-pCDNA3.1-BRCA1-SZ using the TNT T7 coupled reticulocyte lysate system (Promega). Binding assays were carried out with a 10 µl aliquot of ³⁵S-labeled BRCA1-SZ primed lysate and 100 µl (50% slurry of GST-sepharose beads; Amersham Pharmacia) of GST-LMO4 or GST-only protein in GST interaction buffer (150 mM NaCl, 10 mM Tris pH 8, 0.3% NP-40, 1mM DTT, 0.25% BSA and 0.5 mM phenylmethylsulfonyl fluoride) for 2 hours at 4°C. The beads were subsequently washed twice in GST interaction buffer containing BSA, followed by two more washes in GST interaction buffer without BSA. Finally, the bound BRCA1 C-terminal polypeptide was eluted by boiling the beads for 5 min in 30 µl loading buffer and analysed by SDS-PAGE.

Northern blot analysis

Poly(A)⁺ RNA was isolated from human and mouse breast epithelial cell lines cited in previous studies (Douglas *et al.*, 1998) and Northern analysis was performed (Visvader *et al.*, 1997).

Transactivation assays in yeast and mammalian cells

The yeast transcription assay was performed in the yeast strain BJ5462; this was cotransformed with the LacZ reporter plasmid, YepΔ62 (generously provided by P.Vaughan), and pGBT9-BRCA1(AD), and colonies selected on media deficient in leu and trp. These transformants were then additionally transformed with either the pYX212-LMO4 expression plasmid or empty vector, and were selected on media lacking ura, trp and leu. β-galactosidase activities were determined using the o-nitrophenyl-β-D-

- 69 -

galactoside (ONPG) liquid culture assay following standard protocol (CLONTECH Yeast Protocols Handbook).

Kidney embryonal 293T cells were transiently transfected (6-well plates) with the indicated plasmids: 0.5 µg pG5CAT (CLONTECH), 1 µg pCMV-Gal4b-BRCA1-AD or 1 µg Gal4b parental vector, and either 2 µg Flag-EF1α-LMO4 or 2 µg empty control vector using the calcium phosphate precipitation method. CAT activity was determined using the CAT Elisa system (Roche Boehringer) and was normalised against protein concentration, as determined by the Bradford assay (BioRad).

EXAMPLE 6

IDENTIFICATION OF CTIP AS A LMO4-INTERACTING PROTEIN

We used the yeast two-hybrid system to identify LMO4-interacting proteins in breast epithelium. A screen of 8.4×10^6 transformants of a primary breast adenocarcinoma cDNA library yielded more than 800 colonies. Six hundred and fifty-nine β-galactosidase positive clones were isolated and sequentially screened by yeast colony hybridisation using cDNA probes representing the known LMO4-associated proteins, Ldb1 and DEAF1 (Grutz *et al.*, 1998; Kenny *et al.*, 1998; Sugihara *et al.*, 1998), and known false positives. Of 150 cDNA clones sequenced, several candidate LMO4-interacting proteins were identified. One clone corresponded to the complete coding sequence of CtIP (CtBP interacting protein), which encodes a cofactor originally identified on the basis of its interaction with the transcriptional corepressor CtBP (Adenovirus E1A C-terminal binding protein) (Schaeper *et al.*, 1998).

- 70 -

EXAMPLE 7***IN VIVO* ASSOCIATION BETWEEN LMO4 AND CtIP**

A specific interaction between CtIP and LMO4 was confirmed in mammalian cells.

5 Expression vectors encoding either LMO4, LIM homeodomain proteins, each carrying a Flag- or myc-epitope at the N-terminus, together with an expression vector harboring CtIP (residues 45 - 897), were transiently transfected into 293T cells. Whole cell extracts were analysed using a coupled coimmunoprecipitation/western blot assay. As shown in Fig. 5A, Flag-LMO4 and CtIP were found to specifically associate *in vivo*, in reciprocal
10 co-immunoprecipitation experiments (lanes 1 and 5). CtIP was not detected in immunoprecipitates from cells expressing Flag-LMO4 alone (lane 3) or in those using an isotype-matched control antibody. CtIP coprecipitated with the related LIM domain protein, LMO2 (Rabbitts., 1998) (lane 2), but not with the nuclear LIM homeodomain proteins Lhx1 and Lhx3 (Fig. 5B, lanes 2 and 3), nor with LIM-kinase (LMK1) (Fig. 5B,
15 lane 4). A single LIM domain of LMO4 (amino acids 1 - 82) was found to be sufficient to mediate interaction with CtIP in mammalian cells (Fig. 5A, lane 4).

To test the interaction between endogenous proteins in breast epithelial cells, we generated a rabbit polyclonal antiserum against full-length mouse LMO4. This antiserum
20 recognises a protein of about 17 kDa in breast epithelial cells and in cells transfected with an LMO4 expression vector. Nuclear extracts from HBL100 breast epithelial cells were immunoprecipitated with anti-LMO4 antiserum or control antibody. Immunoblotting with a mouse anti-CtIP monoclonal antibody revealed a specific band of 125 kD (Fig. 5C), thus confirming the *in vivo* association between native CtIP and LMO4 proteins.

25

EXAMPLE 8

COEXPRESSION OF LMO4 AND CTIP IN BREAST EPITHELIAL CELL LINES AND SUBCELLULAR LOCALISATION OF LMO4

5 Both the LMO4 and CtIP genes have been reported to be expressed in a number of different tissues and cell types (Kenny *et al.*, 1998; Sugihara *et al.*, 1998; Wong *et al.*, 1998). We surveyed their expression in a panel of breast epithelial cell lines, the majority of which were derived from human breast cancers but also included immortalised human cells (184). Northern analysis revealed CtIP RNA (3.6 kb) levels were relatively uniform
10 while expression of the LMO4 transcripts (1.8 and 2.3 kb) varied dramatically (Fig. 2A). High levels of LMO4 were apparent in a number of human breast cancer cell lines, including BT-549, BT-474, HS-578T, MDA-MB361, T-47D and ZR-75B (Fig. 2), relative to the low levels evident in the immortalised 184 cells (Visvader *et al.*, Submitted).

15 To characterize the subcellular localisation of endogenous LMO4 protein, we analysed LMO4 expression in T-47D breast epithelial cells by indirect immunofluorescence using a rat anti-LMO4 monoclonal antibody. Endogenous LMO4 was found to be localised to both the nucleus and cytoplasm (Fig. 6B). These data are consistent with those reported
20 for cells transfected with LMO4 (Kenny *et al.*, 1998; Sugihara *et al.*, 1998).

EXAMPLE 9

LMO4 INTERACTS WITH TWO DISTINCT DOMAINS IN CTIP

25 The function of CtIP is not known but it appears to serve as a cofactor for several nuclear regulatory proteins, including BRCA1, Adenovirus E1A C-terminal binding protein (CtBP), Retinoblastoma (Rb) and p130 pocket proteins (Fusco *et al.*, 1998; Li *et al.*, 1999; Meloni *et al.*, 1999; Schaeper *et al.*, 1998; Wong *et al.*, 1998; Yu *et al.*, 1998). In

- 72 -

addition to the defined regions that interact with these proteins, CtIP contains two potential leucine zipper domains (120 - 141 and 740 - 761) (Fusco *et al.*, 1998), depicted in Fig. 7A. To delineate the domains within CtIP that mediate interaction with LMO4, a series of CtIP deletion mutants (Fig. 7A), each linked to an N-terminal HA-epitope tag, were co-transfected with Flag-LMO4 into 293T epithelial cells. As shown in Fig. 7B, HA-CtIP (45 - 371), HA-CtIP (371 - 897), HA-CtIP (45 - 897) and HA-CtIP (620 - 897) proteins could associate with Flag-LMO4 (lanes 2, 4, 5, and 6). In contrast, neither the HA-CtIP (59 - 320) nor HA-CtIP (281 - 620) mutants were immunoprecipitable by an anti-Flag antibody (lanes 1 and 3, respectively). Thus, there are apparently two regions within CtIP that can mediate interaction with LMO4: a small domain at the N-terminus (residues 45 - 59) and a C-terminal region (residues 620 - 897) which encompasses a putative leucine zipper motif (Fig. 7A). These regions are distinct from those that associate with CtBP (amino acids 392 - 396), Rb (153 - 157) and BRCA1 (133 - 369) (Fusco *et al.*, 1998; Meloni *et al.*, 1999; Schaeper *et al.*, 1998; Yu *et al.*, 2000; Yu *et al.*, 1998).

EXAMPLE 10

LMO4 ALSO INTERACTS WITH THE BREAST AND OVARIAN TUMOR SUPPRESSOR BRCA1

Since CtIP was recently demonstrated to interact with the breast tumor suppressor BRCA1 (Li *et al.*, 1999; Wong *et al.*, 1998; Yu *et al.*, 1998), we investigated whether LMO4, CtIP and BRCA1 could participate in a multiprotein complex. Both CtIP and LMO4 were co-immunoprecipitated with an anti-myc antibody from 293T cells expressing myc-tagged BRCA1, Flag-tagged LMO4 and CtIP (Fig. 8A, lane 2). This finding revealed that all three proteins can form a stable multiprotein complex *in vivo*. The presence of exogenous CtIP was not necessary for immunoprecipitation of LMO4 by the anti-myc antibody, as shown in Fig. 8A (lane 1). BRCA1 mutants P1749R, M1775R

- 73 -

and Y1853Δ were capable of interacting with LMO4 (data not shown), although they do not associate with CtIP (Yu *et al.*, 1998). Taken together, these results raise the possibility that LMO4 might directly associate with BRCA1.

- 5 To further examine this interaction in breast epithelial cells, a rat anti-LMO4 monoclonal antibody was used to immunoprecipitate proteins from HBL100-derived nuclear extracts. Endogenous BRCA1 was immunoprecipitated by the LMO4 antibody, but not with control antibody (Fig 8B). This result confirms an *in vivo* association between LMO4 and BRCA1 and, moreover, demonstrates that this interaction occurs between native proteins
- 10 in breast epithelial cells. Immunoblotting of anti-LMO4 immunoprecipitated protein with anti-CtIP followed by anti-BRCA1, yielded a faint band of 220 kD, corresponding to BRCA1 (data not shown). Thus it appears likely that LMO4, BRCA1 and CtIP have the potential to form a native complex in breast epithelial cells.
- 15 We investigated whether the nuclear adaptor protein Ldb1, which binds LMO4 and other LIM proteins with high affinity (Grutz *et al.*, 1998; Kenny *et al.*, 1998; Sugihara *et al.*, 1998), could also participate in the multiprotein complex. Ldb1 could be immunoprecipitated from cells transfected with plasmids encoding CtIP, BRCA1, LMO4 and LDB1 using an anti-CtIP antibody (Fig. 8C). Therefore, all four proteins have the
- 20 potential to form a stable complex *in vivo*.

EXAMPLE 11

THE BRCT DOMAIN OF BRCA1 INTERACTS WITH LMO4

- 25 The C-terminal 335 amino acids of BRCA1 (SZ fragment, residues 1528 - 1863) were sufficient to mediate interaction with LMO4 (Fig. 9A) in transfected cells. This region encompasses two tandem BRCT motifs that are required for BRCA1 function in the suppression of tumorigenesis (Li *et al.*, 1999; Wong *et al.*, 1998; Yu *et al.*, 1998). We

- 74 -

confirmed the association between LMO4 and BRCA1 using an *in vitro* binding assay. Full-length LMO4 expressed as a glutathione-S-transferase (GST) fusion protein, immobilised on glutathione sepharose beads, was incubated with *in vitro* translated, ³⁵S-methionine-labeled BRCA1-SZ polypeptide (1528 - 1863). As shown in Fig. 9B, BRCA1 specifically associated with GST-LMO4, but failed to interact with GST alone. Further, the SZ portion of BRCA1 linked to the Gal4-activation domain interacts with GBT9-LMO4 in yeast Hf7c cells, providing evidence that BRCA1 and LMO4 associate directly. Thus, the BRCT domains of BRCA1 mediate direct binding to both LMO4 and CtIP.

EXAMPLE 12

LMO4 REPRESSES THE TRANSCRIPTIONAL ACTIVITY OF BRCA1

The BRCA1 C-terminal (BRCT) domain has previously been shown to activate transcription when recruited to a promoter via a heterologous DNA-binding domain (Chapman *et al.*, 1996; Monteiro *et al.*, 1996). Further characterisation of the C-terminus has revealed that there are two adjacent activation domains, AD1 (residues 1293 - 1558) and AD2 (residues 1560 - 1863), which together constitute the AD domain of 571 amino acids (Hu *et al.*, 2000). Functional synergy occurs between AD1 and AD2 in both yeast and mammalian cells, such that the AD region is a stronger transcriptional activator than either domain alone (Hu *et al.*, 2000). To explore the effect of LMO4 on activation of transcription by BRCA1, we initially used a yeast-based transcription assay with the C-terminal AD region of BRCA1 fused to the Gal4 DNA-binding domain. This plasmid was introduced with either a LMO4 yeast expression vector or an empty vector into the BJ5462 yeast strain, harboring a LacZ reporter plasmid. BRCA1-AD was a potent transactivator, inducing transcription 400- to 600-fold over basal Gal4 DNA-binding activity (Fig. 10A). This activation was markedly repressed by LMO4, which resulted in an 80% reduction in the level of activation by BRCA1-AD (Fig. 10A). The expression of

- 75 -

BRCA1-AD was unaffected by the presence of the LMO4-encoding plasmid (Fig. 10A, lower panel).

To confirm the findings of the yeast transcription assay, we performed a similar study in mammalian 293T cells. BRCA1-AD tethered to the Gal4 DNA-binding domain induced transcription of the Gal4-CAT reporter gene by 3- to 4-fold over the basal activity of the Gal4-DNA-binding domain alone (Fig. 10B). Consistent with the findings above, LMO4 exerted a negative effect on transactivation by BRCA1 (approximately 3-fold), returning transcriptional activity to the basal level observed with the Gal4 DNA-binding domain (DBD) alone.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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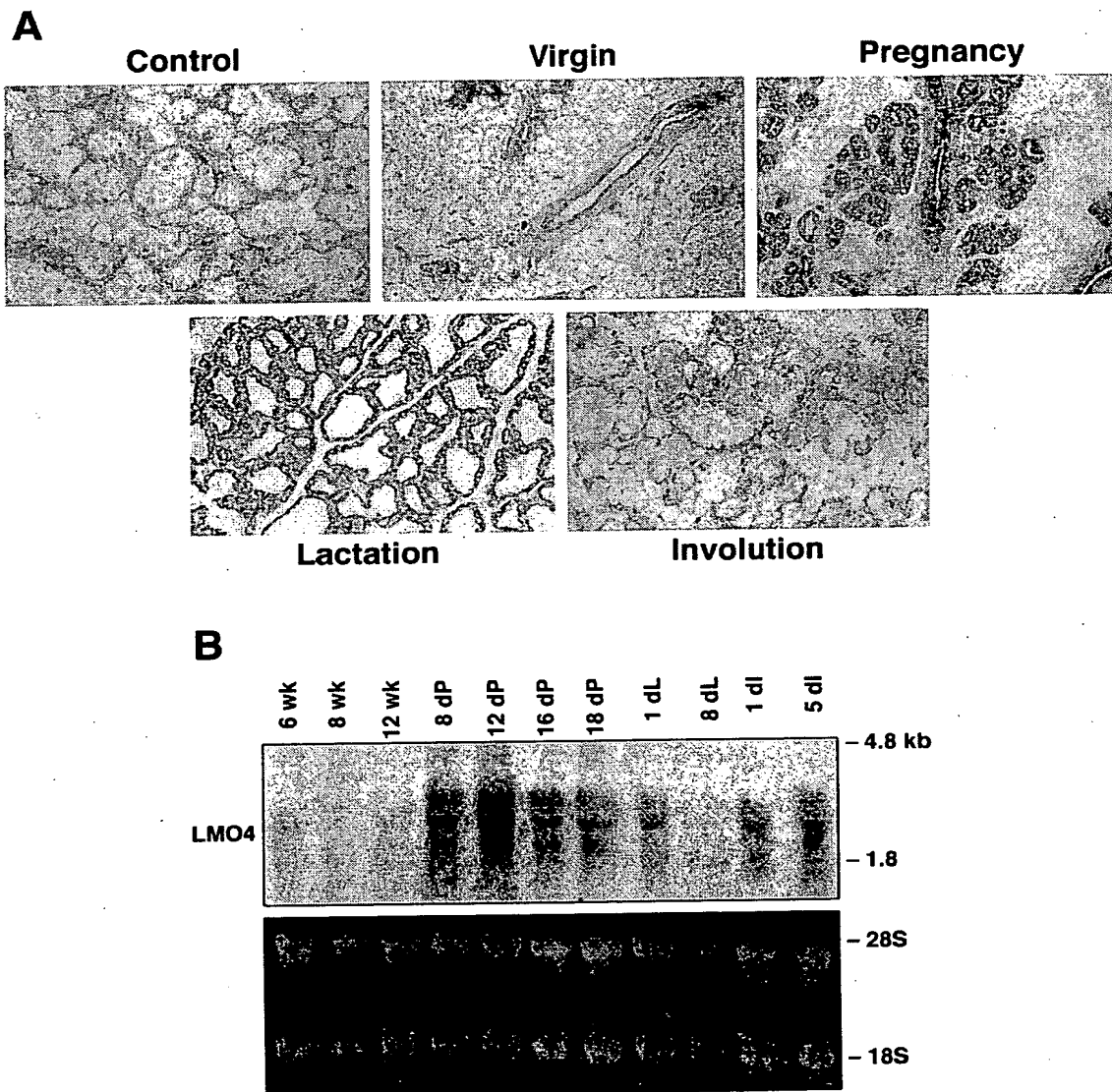
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Dated this 12th day of September, 2001

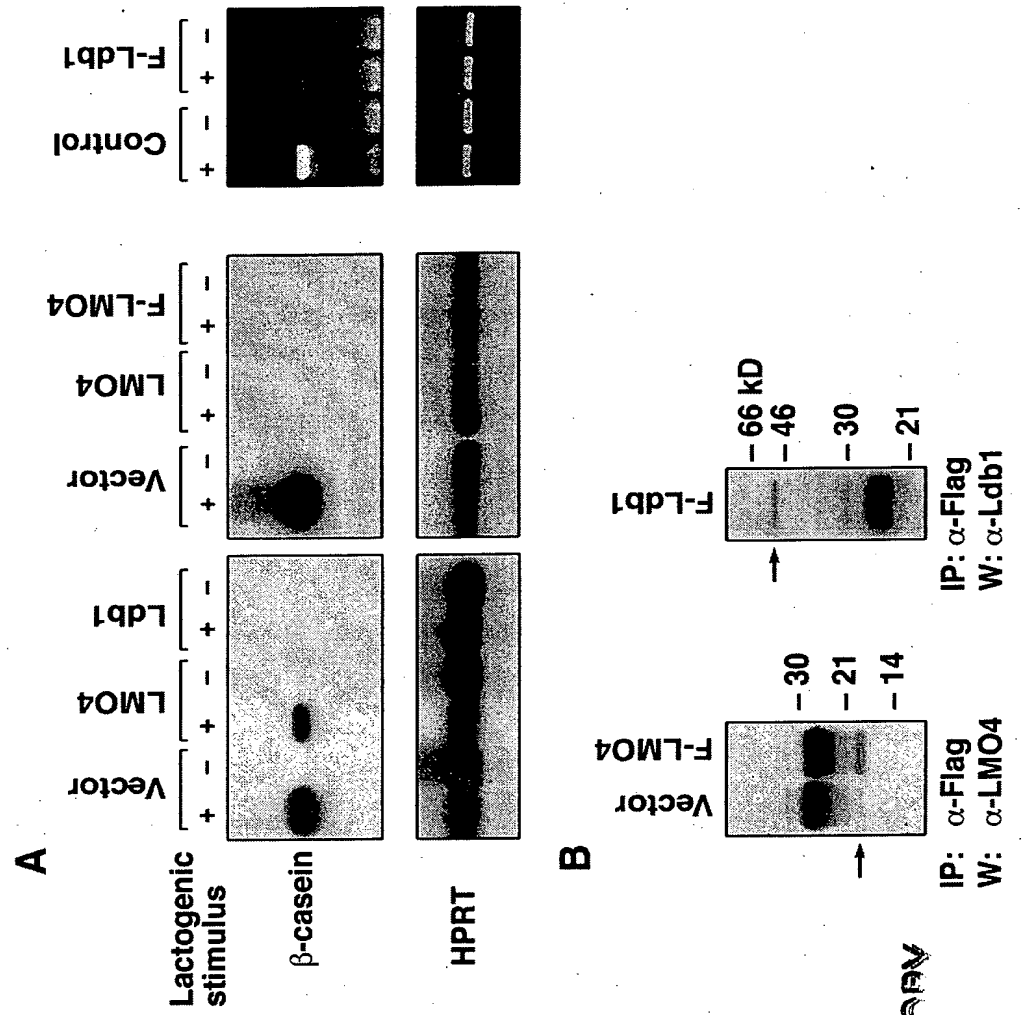
The Walter and Eliza Hall Institute of Medical Research
by its Patent Attorneys
DAVIES COLLISON CAVE

Figure 1



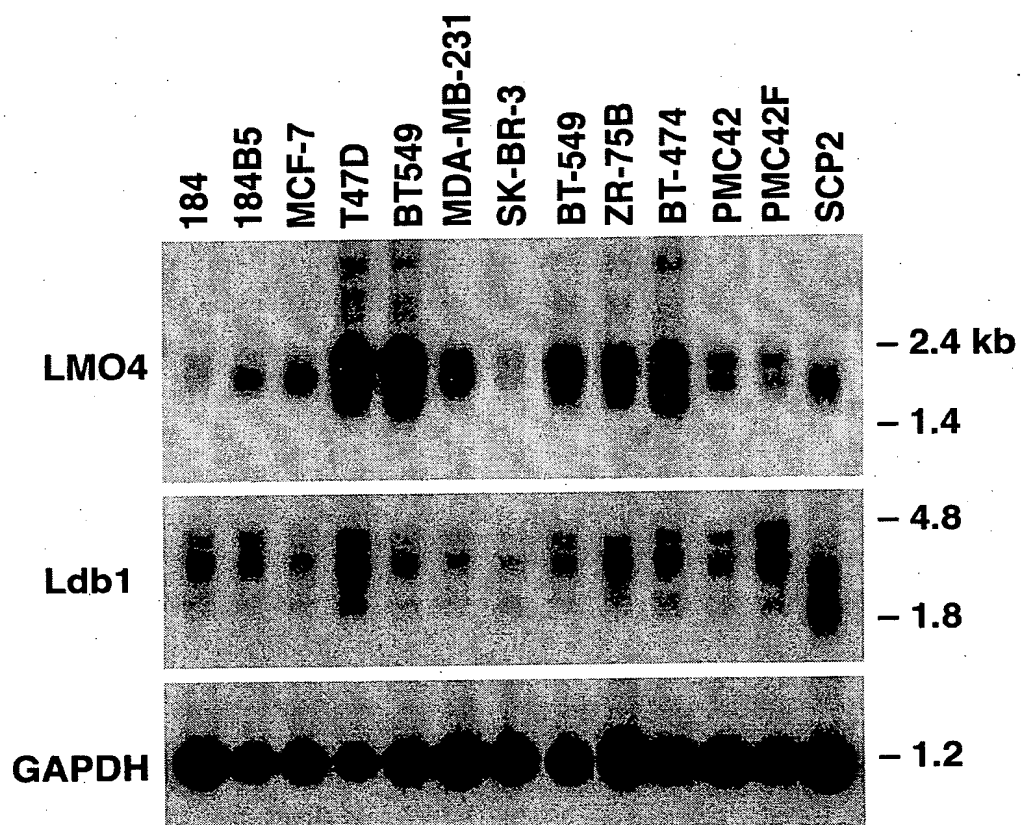
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Figure 2



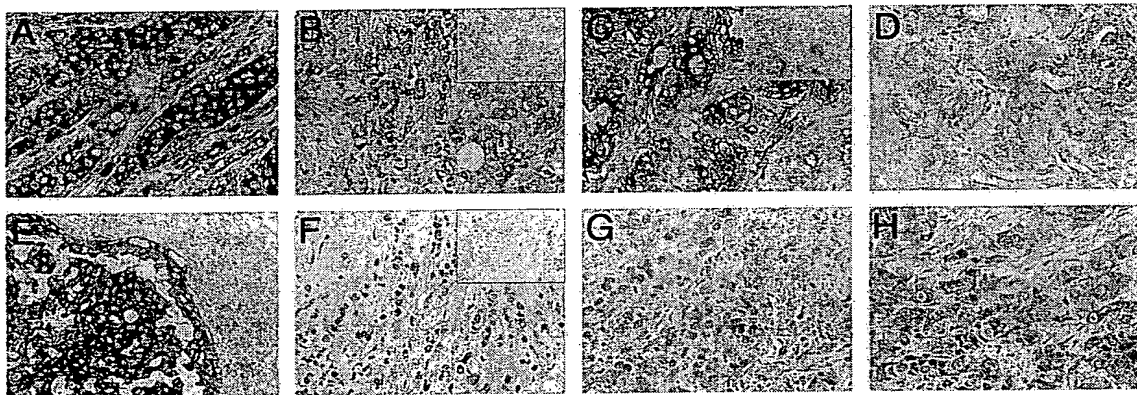
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Figure 3



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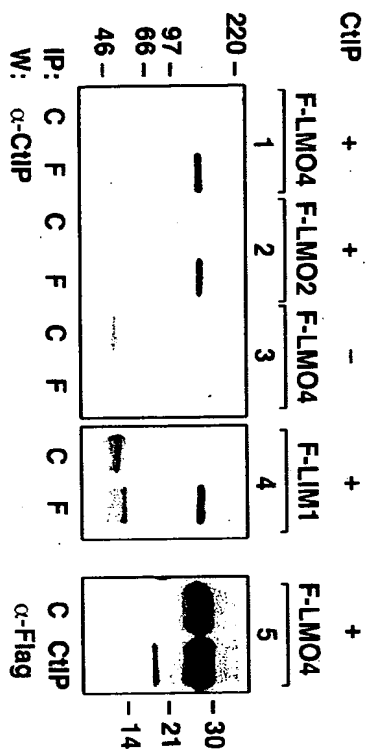
Figure 4



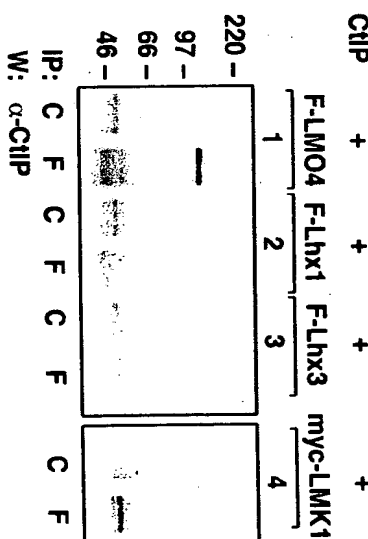
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Figure 5

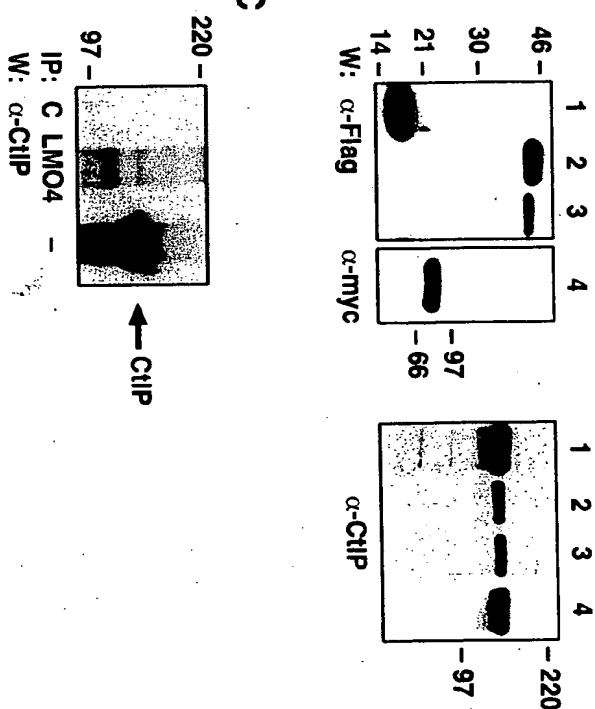
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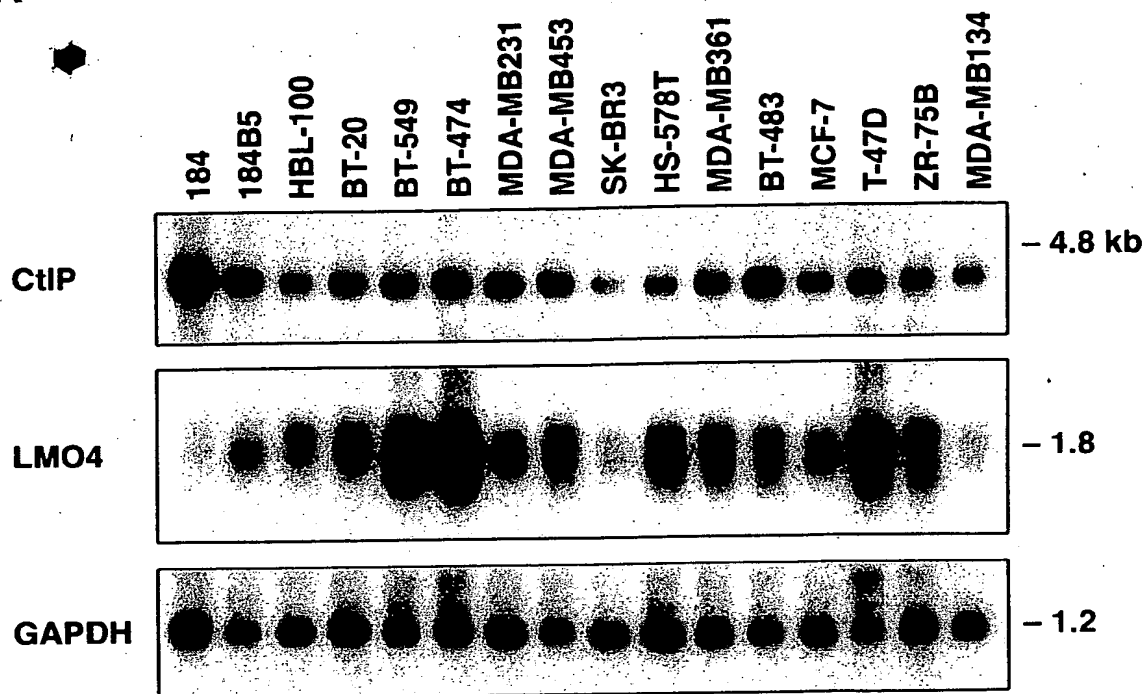
B



C



A



B

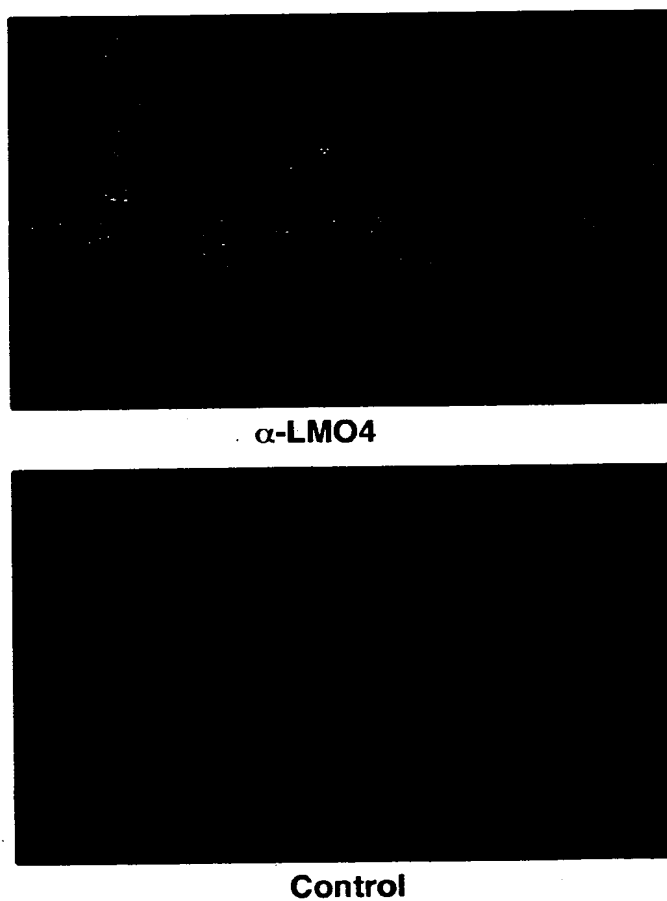


Figure 6

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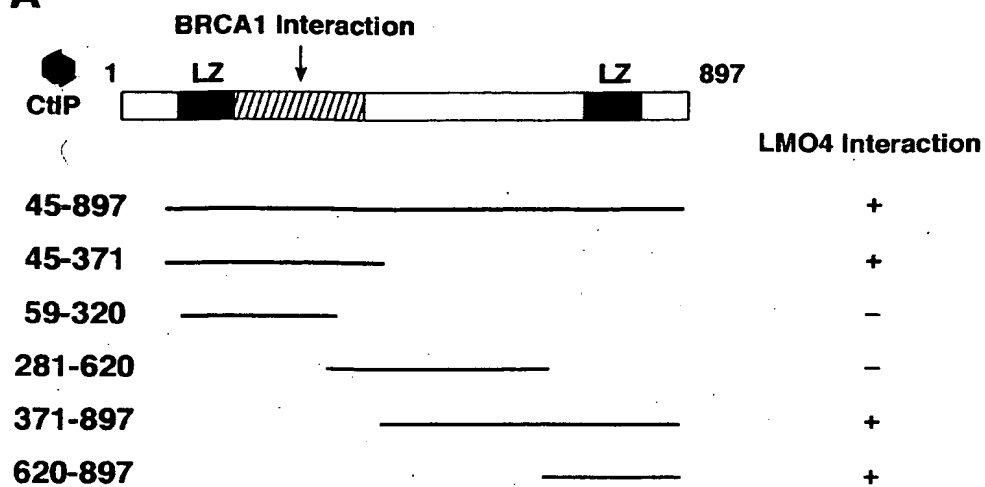
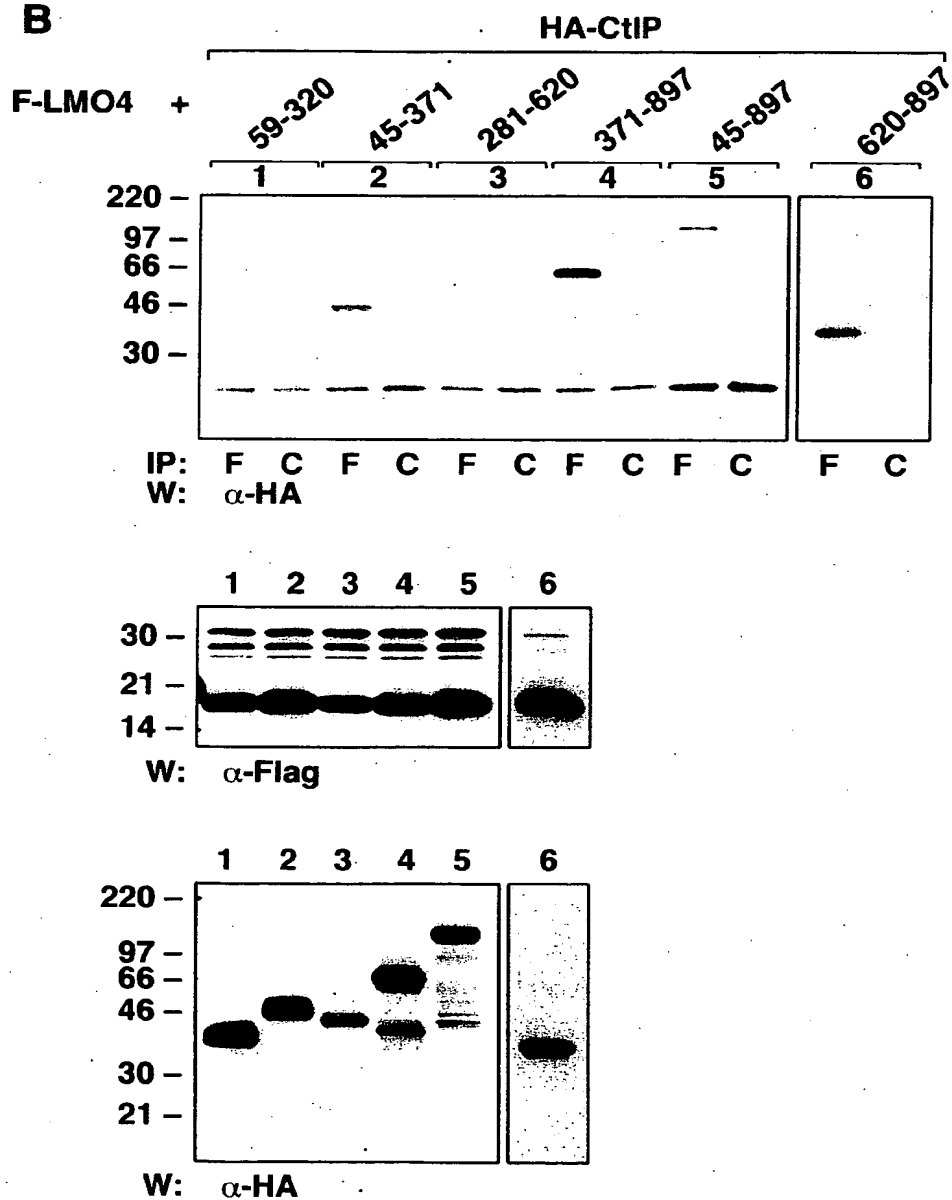
A

Figure 7

B

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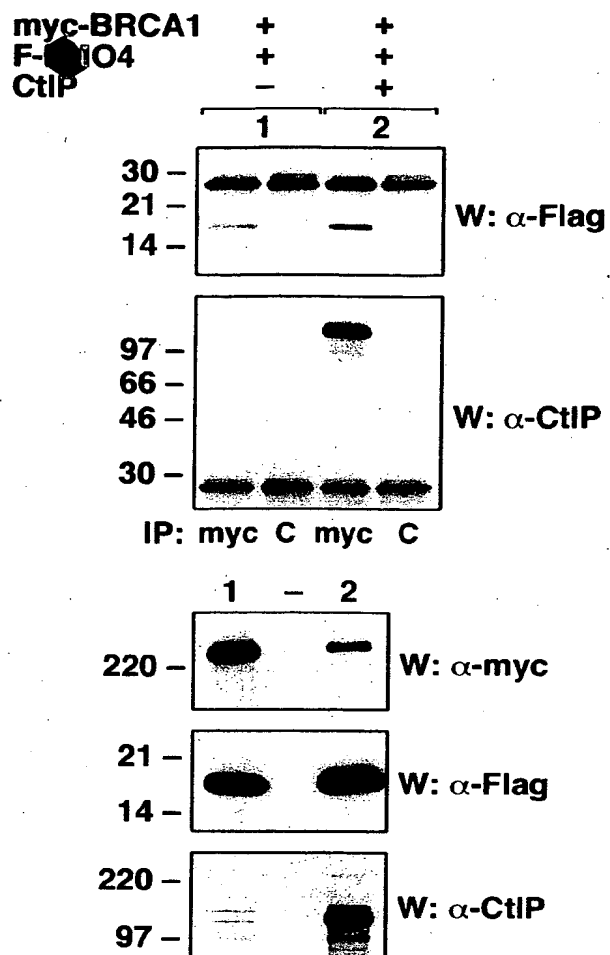
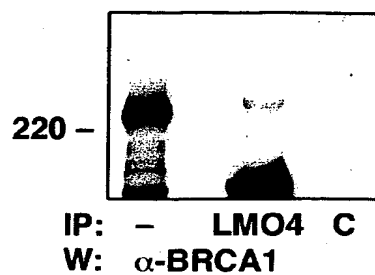
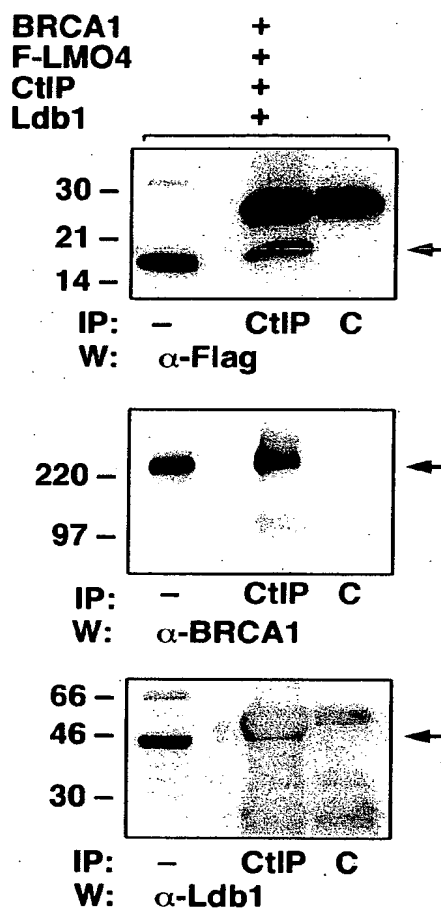
A**B****C**

Figure 8

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A

HA-BRCA1(SZ) +
F-LMO4 +

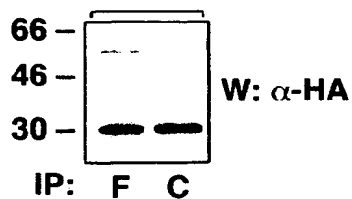
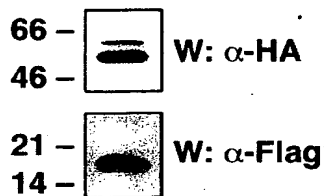
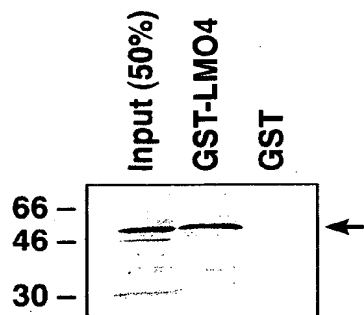


Figure 9

**B**

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